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Diagnostic bioch.

DIAGNOSTIC
BIOCHEMISTRY

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Quantitative Distributions of Body Constituents and Their Physiological Interpretation

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FOREWORD

The present volume has grown out of past teaching at several levels of medical education; it may be recommended either as a supplementary text for a first course in biochemistry that penetrates vigorously into the diagnostic area, or for an advanced course for students who have had the more ordinary first course. At the same time the needs have been kept in mind of the physician who wants to reconsider the subject briefly from the ground up to improve his comprehension of biochemistry. The text may also serve to supplement hematology and clinical microscopy texts for instruction in laboratory diagnosis. Finally, the current tendency to explore all sorts of partitions and recombinations of medical education appears to create places for smaller presentations of special subjects like the present one.

In line with these objectives an attempt has been made to limit the present discussions to what may be more or less central areas of each subject, and to leave the discovery of some of the peripheral information to the student and to assign him questions that will cause him to manipulate the central ideas. The author has in mind that each student be assigned to think out some problem or to uncover some information that is available in the literature, and to communicate his result to his colleagues by a discussion, paper, or brief talk. Sometimes our students receive only one such assignment per semester; in other courses (for example, advanced elective courses) much of the didactic time may be spent with such problems. These assignments, some of which are proposed at the end of each chapter, require that the student know how to find papers in the original and derivative literature by subject or by author. Accordingly a preliminary discussion of library techniques has been included at the end of Chapter 1. In this way a simple course in library use is integrated with the central subjects.

Students are often deprived of the pleasure and benefits of the discovery for themselves of facts and relationships from various sources. Also they may be isolated somewhat from the actual process of the advance of knowledge, and from practice of their critical faculties, by the exclusive use of thoroughly prepared textbooks, lectures, and reviews (and now even curricula). As teachers we are supposed to *open doors* to the student. A question often holds the door open better than an answer (or a lecture), and at the same time may help the student to learn to find his own doors. One would suppose that such self-teaching, even if advantageous, would be inefficient; we are continually surprised that it is not.

Accordingly this book is written not only to serve as a sole source of information but also to guide the student to the library and to other counsel for some of the ancillary ideas. Obviously this arrangement gives priority to the needs of the medical student rather than to those of the medical scientist who may be looking for an extensive and profound review.

Indulgence needs to be requested in connection with a few of the assignments that ask the student to find small slips or inconsistencies, oral or typographic, in relation to case reports. The intent is to stimulate careful and critical reading and certainly not to lift eyebrows. Two types of library assignment not included in the lists may occasionally be useful: a student challenge of any account presented here, and a description by the student of a specific analytical procedure. In general, laboratory procedures have been discussed in detail only when required for an exposition of the significance of the results.

Acid-base balance is presented here in terms consistent with the chemical training that the entering medical student of today brings with him. Only those who are teaching first courses in biochemistry realize what a strain the student undergoes in inverting his thinking to some of the historic expositions. At the same time, however, the support to this subject provided by analyses for fixed anions and cations is also developed.

The list of references in the Appendix has been curtailed somewhat to encourage a conventional mode of attack on the library problems included here, and also in recognition of the extensive bibliographies to be found in some of the treatises listed in Chapter 1.

The author wishes to acknowledge his indebtedness to his mentor, A. Baird Hastings, whose teaching and inspiration are reflected here. A debt of inspiration is also owed to Drs. Donald D. Van Slyke and James L. Gamble. I also want to acknowledge the suggestions and critical reading of all or portions of the manuscript by the following colleagues: Drs. Joseph P. Chandler, Adam A. Christman, Minor J. Coon, Stefan Fajans, Armand J. Guarino, Raymond Knauff, Lawrence Louis, Muriel Meyers, Saul Roseman, and David H. P. Streeten. Sketches and charts by Mr. Jamie Ross, and checking of all references by Mr. David Kronick, are also gratefully acknowledged. I am also particularly indebted to Dr. Benjamin Castleman and Dr. Joseph Garland for their generous permission for me to use the Case Records of the Massachusetts General Hospital, as published in the *New England Journal of Medicine*, for many of the library problems included.

H. N. C.

Ann Arbor, Michigan
March 1959

CONTENTS

1. Concentration 3
2. How the Hydrogen Ion Distributes Itself 16
3. The Distribution of Sodium and Chloride 31
4. Potassium Distribution 46
5. Calcium and Phosphate Distribution 64
6. Gas Transport 79
7. Respiratory Influences on Hydrogen-Ion Distribution 97
8. Renal Disposition of the Hydrogen Ion 112
9. Endogenous Production and Consumption of Hydrogen Ions 122
10. Anion-Cation Balance 128
11. Small Nitrogen Compounds—Distribution of Amino Acids 135
12. Distribution of Other Small Nitrogen Compounds 146
13. Circulating Proteins: Normal and Abnormal 157
14. Lipid Transport 176
15. Circulating Carbohydrate 185
16. Iodine and the Circulating Thyroid Hormones 198
17. Steroid Hormone Transport 207
18. Heme Precursors and Products 224
19. Getting Useful Chemical Laboratory Results 233

BIBLIOGRAPHY 247

APPENDIX. Examination Items in Diagnostic Biochemistry 259

INDEX 283

DIAGNOSTIC
BIOCHEMISTRY

1

CONCENTRATION

Convenient references for the student of diagnostic biochemistry are the Interne's Handbooks, which give the normal values for a large number of blood and urinary constituents. Figure 1.1 illustrates a sample page from such a book. The information contained in the handbook will permit you to decide whether a laboratory result

Constituent	Material *	mg./100 cc. (mg. %) (or as noted)
Albumin	S	3.5–5.5 g./100 cc.
Amylase	P, S	70–200 units (Somogyi)
Ascorbic acid	P	0.4–1.0
Calcium	S	9–11 (4.5–5.5 milliequiv./liter)
Carbon dioxide (combining power)	S	56–65 vol. % (25–30 milliequiv./liter)
Carotenoids	S	100–500 Int. Units/100 cc.
Chloride	S	350–390 (100–110 milliequiv./liter)
Chloride, as NaCl	P	550–620
Cholesterol, total	S	110–300
Cholesterol, free	S	35–90
Cholesterol, esterified	S	75–210
Creatine	B	3–7
Creatinine	B	1–2
Fibrinogen	P	200–600
Globulin	S	1.5–3.4 g./100 cc.
Glucose	B	80–120
Glutamine	P, S	0–2

* B—Whole blood. P—Plasma. S—Serum.

Fig. 1.1 Excerpt from *A Pocket Book of Normal Laboratory Values . . .*, 1957, prepared by Smith, Kline & French Laboratories, Philadelphia.

you have obtained on a patient is *high, low, or normal.* You can then assimilate this finding and other relevant observations concerning the patient. If appropriate information already has been stored by you, you can come up with one or more answers, along with a feeling for the likelihood for each—perhaps also a suggestion of another line of inquiry that will increase your degree of certainty.

My question is: "What kind of information about the meaning of laboratory results will you store?" Will it be of these types: high blood sugar = diabetes; high NPN = renal insufficiency; low chloride = saline needed? Will the number 45 be an arbitrary numerical index that tells you have entered the region of elevated NPN or of elevated cerebrospinal fluid protein? Or will 45 mg. of NPN per 100 ml. give you a picture of a certain total concentration of an assortment of real substances that have reached this total level because of changes in the balance between their collective entry and exit from the solution which you have sampled? Perhaps, because many of the *morphologic* changes of disease are accepted more or less empirically, we are inclined to think of changes of *chemical concentrations* as empirical signs of disease and to overlook their subtle and dynamic character.

A simplified mental shorthand, like *NPN above 45* = renal insufficiency, may be convenient and safe for the experienced, but most of such equivalencies are "booby-trapped" for the unwary. For safety these notations need to be attended with a whole series of "feedback" qualifications (conscious or unconscious) arising from other information about *the patient* and about *this particular laboratory analysis.*

A colleague has suggested that the chemical laboratory should report only a percentile score for every analysis, for example *90* for the NPN to mean that only 10% of persons have a higher level. Thus one could have both a total serum protein and a protein-bound iodine of 68! The statistical aspect is protected, but the reality represented by the analysis becomes even more remote. We shall see subsequently that if a patient has a serum CO_2 of 10 millimoles per liter, a normal serum chloride *for him* would be 120 milliequivalents per liter rather than the usual value of about 103. What benefit would you receive from being told that this patient's chloride analysis lies at the 99.99 percentile? To retain control of such feedback corrections, you must have the concentration values *per se.*

Concentration and Rate. We must first reconsider the concepts of concentration and rate. Perhaps we must stop to decide whether we really want to know the *concentration of a substance* or the *rate of a process.* In urinalysis we usually want to know a *rate,* the total amount of a substance excreted in 24 hr. or in some other interval, and we are not directly interested in the concentration. The urinary concentration tests are an exception, for in these the abilities to reabsorb and excrete water are under investigation.

Blood analysis on the other hand ordinarily yields *concentration of the substance,* which may be the most serviceable information. In many cases we are also interested in the total supply of the component; to obtain this we need to know the third dimension, the volume of the plasma or blood or extracellular fluid, or whatever the pool that our concentration measure represents. If we had this information we should know the total blood hemoglobin or the total serum albumin or the total extracellular fluid Na^+, and we should have a much better estimate of the actual deficiency. Despite our emphasis on blood, however, there is scarcely an instance where the circulating blood volume represents even closely the true pool size of a component.

We are so accustomed to the idea that it is the blood or serum *level* which we want, that as a shorthand expression we may ask, "What was the serum albumin? What was the urine calcium?" We take for granted that we shall be understood in one case to refer to a *concentration unit;* in the other, to an *excretion rate.* Note also another type of shorthand whereby the serum alkaline phosphatase activity may be recorded as 11 Bodansky units, when *concentration* (presumably 11 units per 100 ml.) is actually meant.

Preserving the Sense of Reality of Concentration Units. *Five per cent glucose* has an obvious meaning: 5 g. of glucose in 100 ml., or 50 g. per liter. If we know that 1 mole of glucose is 180 g., we can calculate that we have 50/180, or $0.278M$ glucose. A little laboratory experience in preparing solutions makes the *molar* unit as real as the *per cent* unit. Similarly, 0.9% NaCl is 9 g. per liter, or 0.154 molar, or 154 millimolar.

The sense of reality is apt to be broken when we pass over to a Na^+ concentration of 154 *milliequivalents per liter.* Had the term *millinormal* gained acceptance, so that we could say 154 millinormal, half the obscurity would have been avoided. The other complication comes in deciding whether a gram atomic weight of a constituent like Na^+ or Ca^{++} represents *one, two,* or *more equivalents.* If we divide the weight of the solute by its milliequivalent weight, we obtain the number of milliequivalents.

Blood, Plasma, and Serum. Blood is at least a two-phase system, a fact we usually neglect when we state the blood concentration of a substance. The blood concentration is in effect a weighted mean concentration for the two phases. If the solute is restricted to the plasma or to the cells, its real concentration in that phase will be much higher than the average blood level. *Plasma* may be defined as the *extracellular phase* of the blood, or the same fluid modified so as to prevent its coagulation. *Serum* is a fluid produced from plasma by the coagulation reactions. In our discussions we shall encounter this ambiguity: Our analyses usually will be made on *serum,* but when we shall talk about the *circulating fluid* we shall speak properly only of the *plasma.* For example, we may say that the *serum* cholesterol is 210 mg. per 100 ml., but we shall refer to the mode of cholesterol transport in the *plasma.*

Significant Decimal Places. A reported concentration of 4.70 milliequivalents per liter is a claim of accuracy of better than one part in 47. The number should *not* be reported as 4.7 unless one wants to indicate that the uncertainty is still greater. Similarly, a reported cholesterol concentration of 198.8 mg. per 100 ml. represents a claim of a rather unlikely accuracy of better than one part in 199.

Osmolal Concentration. We know that it makes a great deal of difference what molecules are present in the blood or other body fluids. There is one attribute, however, that seems to be provided as well by one solute molecule as by another; that is, *osmotic pressure.* The *total* number of solute particles, whether they be molecules or ions, determines this property.

The *osmolal concentration* (usually in milliosmoles per kilogram of water) is just such an undiscriminating summation of all the particles present in a solution. One could analyze for all the likely components, but not very practically, and add them up, being sure to count $Na^+HCO_3^-$, for example, as two components. More practically one can determine very accurately how much the *freezing point* of serum or of a digestive juice or of urine is lowered from the freezing point of water. If this difference is divided by the *millimolal freezing point depression*, we get the milliosmolal concentration, the freezing point depression being another of the properties (*colligative* properties) that depend only upon the number of particles present.

The physician is so frequently concerned with the much smaller *colloidal* osmotic pressure or *oncotic* pressure that the very great sensitivity of cells and subcellular particles to the *total* osmotic pressure is perhaps overshadowed. Enormous pressures are set up by entering water when a cell is placed in a hypotonic solution, because of the difference in *total* osmotic pressure. Likewise, large amounts of work are required to produce a secretion with a higher or a lower *total* osmotic pressure than the body fluids.

Gas Concentration. We know the blood occasionally may contain, for example, as much as 16.5 g. of hemoglobin per 100 ml.—quite a large mass if separated out on a sheet of filter paper. If we know that 1 mole of oxygen combines with 16,500 g. Hb, or 1 millimole of oxygen with 16.5 g., we can understand that the oxygen capacity provided by the hemoglobin would be 10 millimoles per liter. If a millimole of oxygen under standard conditions is 22.4 ml., this would be 224 ml. per liter, or 22.4 ml. per 100 ml. (22.4 *vol. per cent*).

Suppose, however, you are told that a sample of venous blood has an O_2 pressure of 35 mm. of Hg. This is also in effect a *concentration unit*. You could reproduce this situation by bringing blood into equilibrium with a gas mixture in which the oxygen pressure is 35 mm.; for example, by using 4.6% oxygen in a gas mixture under a total pressure of 1 atm., or 760 mm. Hg. But even after the blood is separated from this gas phase, or even if we are speaking of circulating blood, we can still say it has this O_2 pressure although there is no gas phase present.

You can perhaps make this designation more real to yourself if you stop to imagine that a tiny bubble of gas phase is caused to be formed over a large volume of the blood. In this bubble oxygen would then exert the stated O_2 pressure.

Let us now show that this is really a concentration unit. The oxygen pressure of blood gives us the concentration of physically dissolved oxygen because, according to Henry's law, the concentration of gas dissolved varies directly with the pressure, $C = KP$. If we know K, we can calculate C from P.

When given in milliliter of gas dissolved in 1 ml. of solution at 1 atm. of the gas, K is called α (the Bunsen solubility coefficient). The value of α for blood is 0.55 for CO_2 at 38°. Hence the physically dissolved CO_2 in blood at $P_{CO_2} = 40$ mm. Hg is $0.55 \times 40/760 \times 1000 = 29$ ml. per liter. Oxygen is less soluble, α being 0.024. Therefore, at $P_{O_2} = 100$ mm., by a similar calculation 0.32 vol. per cent will be dissolved.

The concept emphasized here is that we can record the concentration of physically dissolved oxygen in terms of O_2 pressure quite as well as in other concentration units. We cannot, of course, calculate the amount of *bound* oxygen from the P_{O_2} unless we have further information.

Partition. The foregoing concept can be extended one step further. During ether anesthesia we may know the *ether pressure* of the alveolar air with which the blood is approaching equilibrium. Or we may instead determine the *ether concentration* of the blood. Or conceivably, we may know the concentration of ether in a third phase; for example, the *fat droplets* of adipose cells. At an equilibrium distribution, either one of these should be equally successful in measuring the ether level of the organism. The ether level at every site in the organism may be expected to bear a definite relationship to that in the air or the blood if a steady state has been achieved.

This concept may be examined for applicability to the problem of the concentration of any metabolite or drug at a particular site. The cellular concentration may be *higher* or *lower* than that in the blood plasma, but these concentrations are usually related. For example, the various amino acids are at much higher levels in the cells of the organism than in the extracellular fluid. Nevertheless, when the plasma level is artificially elevated for a short period of time, a corresponding elevation occurs in the cellular levels. Even before we know why this occurs, we can recognize the validity of this relationship and use it. Such recognized *partitions* between body compartments, where applicable, give far more value to serum analyses than they otherwise would have.

Sodium ions are largely confined to the extracellular compartment of the body; a rise of the extracellular Na^+ level, however, accelerates the movement of Na^+ into the cells, and the cell level must rise until sodium extrusion matches the rate of sodium entrance. Conversely, a fall of the extracellular K^+ level will cause K^+ loss from the cells. In these cases influences of changes of extracellular level are transmitted to the interior of the cell.

Examples of total lack of correspondence between cell levels and the circulating levels of a constituent are, of course, not hard to find. We do not expect to estimate tissue levels of enzymes or of triglycerides by serum analyses: Enzymes usually are confined to the cell of origin by their nature; triglycerides are highly insoluble, and a large fat globule in an adipose cell probably has no more tendency to dissolve or to enter the plasma than a small one.

Other substances become partially bound to cell constituents; the free form may be uniformly distributed, but the *apparent* concentration in cell will be increased by the amount of the bound form. If the amount of the binding agent is not too variable or the binding is not too stable, we may nevertheless note tendencies of the plasma level and tissue level to change together.

Such a substance as Cu^{++}, however, combines very stably with so many cellular and extracellular constituents that the free concentration probably is extremely small. Therefore Cu distribution presents a very complex problem, undoubtedly governed by the distribution, net movements, and affinities of the agents that bind it.

We must also understand that *large* amounts of a component may be transferred from one place to another without its appearing in appreciable concentrations in an *intermediate phase* (for example, the blood) or in fact without its appearing in significant concentrations in *any* of the phases. Thus H^+ in large amounts may be transferred from the cells to the blood and into the urine without appearing in substantial concentrations in any of these places. This is the fact that has confounded many students in interpreting neutrality control.

This fragmentary exploration of *partition* or *distribution* is preliminary to the discussions to follow. It seeks to emphasize how much we depend upon distributional concepts for each substance in clinical chemistry. We shall now proceed in subsequent chapters to consider for a number of illustrative substances the principles governing their distribution; these principles, which are the same in health and in disease, govern the interpretation of the changes in solute levels in blood and other fluids.

Rate Measurements. Frequently our measurements of a serum constituent are not in reality intended to reflect the *concentration at some critical* site; instead they are intended to detect a *changed rate* of some process or reaction; for example, a *rate of formation* or a *rate of excretion* of the substance. Concentration measurements are not well suited for this purpose. Consider, for example, the number of factors influencing a glucose tolerance test, in which we try ordinarily to discover how quickly sugar is utilized, by following the blood sugar level after an oral dose. Basic biochemical research, by using isotopic tracers, has greatly decreased its dependence upon the changes of *concentrations* of substances in complex systems by substituting direct measures of *rates* of their entrance, exit, formation, or destruction. These rates are known as *fluxes*, measurements of change in one direction unobscured by opposed reactions. Note that *flux* means *unidirectional rate*, so that we do not speak of a *flux rate* but merely of a *flux*. Presumably, similar isotope tracer techniques can yield us a new diagnostic chemistry, by which we can measure differentially, for example, the rates of bilirubin formation and elimination, rather than having to be satisfied with a net figure based upon a concentration analysis. Some methods of this type are already in use; for example, the thyroid uptake of radioactive iodine. The interpretation of the results will be freed from many of the complexities to be discussed here if flux measurements become sufficiently safe and simple to displace determinations of concentration. For example, if we learn that bilirubin formation is occurring at a rate of 1 g. per day, this is very straightforward information. But if we are to interpret the finding that the serum indirect bilirubin is 12 mg. per 100 ml., we are faced with a much more complex conceptual problem. This book seeks to assist with this problem.

On the other hand, flux measurements are not always simple or satisfactory because flux may occur in both directions without any net change. Measurements of *net change in one direction* are often what we really want; neither concentration nor flux measurements may furnish this value.

A Note on the Recovery of Stored Information. This volume is intended to point out to the student the existence of some ideas and information in the area of biochemical diagnostic procedures without trying to transmit the whole of this information directly to him. Instead, each brief presentation is followed by an assorted list of problems and questions that have been designated, not always accurately, as *library problems.* If each of a group of students will take responsibility for investigating one of these (omitting assignments inappropriate to the purpose of the particular course, and supplementing freely), perhaps a productive discussion or series of oral or written presentations can result.

Where the student needs to supplement his information in dealing with these assignments, he may find such information in a number of places. None are more convenient than *textbooks,* but these predigested summaries readily produce dependence or even addiction. The present textbook does not seek to increase confidence in textbooks, because the textbook writer suffers from the disadvantage of an inherent time lag, averaging probably not less than five years.

Furthermore the student may frequently find *reviews* of a given subject in *monographs,* in *symposia,* or in various *review journals* or *review sections* of other journals. These usually are better for having been prepared by an investigator in the area concerned, and they can lead the student back to the experimental details themselves. But here, too, the time lag may be serious.

Finally, the student needs to become familiar with that primary source, the *research paper,* laying forth the actual evidence along with its interpretation by the investigator. The time lag for the research paper averages perhaps a year from laboratory to print. In this source one can examine for himself the evidence, economical or inefficient, and the argument, tenuous or compelling. Such papers also usually summarize the current situation as seen by the author and lead one backward over the trail of advance.

Finding Scientific Articles. To begin a literature search, however, one ordinarily uses a type of periodical that *lists* published papers or one that *abstracts* the papers. The former should inherently keep more nearly current, but the latter usually permits faster winnowing by the searcher. Probably the most valuable listing journal at present is the *Current List of Medical Literature,* issued by the Armed Forces Medical Library. *Quarterly Cumulative Index Medicus* is also valuable, although unfortunately it has fallen about two years behind schedule.

Among the numerous abstracting journals, *Chemical Abstracts* should prove highly serviceable for the problems included here. The *subject indices* covering the last full calendar year of *Chemical Abstracts* usually appear as much as a year late, whereas *author indices* appear in the biweekly issues and in the December 10 issue with an insignificant time lag. Until the subject indices appear, abstracts of recent papers by unidentified authors can be found only by screening an appropriate section; for example, 11B, *Biochemical Methods,* in each issue.

In making a careful investigation of a current subject of research, the student will do well also to examine issues of at least a half dozen relevant journals of the past twelve months. These issues probably will not have yet been fully abstracted. Finally, if he wants the luxury of knowing entirely current thought, he must rely at least partially on oral research presentations. This is not to imply that the most recent conclusions are *always* the best. Among the journals that will be most frequently helpful for the present assignments, we may mention the *American Journal of Medicine, Biochemical Journal, Clinical Chemistry, Journal of Biological Chemistry, Journal of Clinical Endocrinology and Metabolism, Journal of Clinical*

Investigation, Journal of Laboratory and Clinical Medicine, New England Journal of Medicine, Pediatrics, and *Proceedings of the Society for Experimental Biology and Medicine.* In connection with the problems derived from the Case Records of the Massachusetts General Hospital, a list of methods used and normal values obtained (*New England J. Med.,* **254,** 29 [1956]) should be examined. The following recent publications probably will also be found particularly helpful:

1. Thompson and King, *Biochemical Disorders in Human Disease,* Academic Press, New York, 1957.
2. Bodansky and Bodansky, *Biochemistry of Disease,* Second Edition, Macmillan, New York, 1952.
3. Gamble, *Chemical Anatomy, Physiology and Pathology of the Extracellular Fluid,* Harvard University Press, Cambridge, 1954.
4. *Hormones in Blood,* Ciba Foundation Colloquia in Endocrinology, vol. 11, Boston, 1957.
5. Elkinton and Danowski, *The Body Fluids,* Baltimore, 1955 (includes valuable bibliographies).
6. Bland, *Disturbances of Fluid Balance,* Philadelphia, 1956.
7. *Methods of Biochemical Analysis,* D. Glick, Editor, New York (annual publication).
8. Ham, *A Syllabus of Laboratory Examinations in Chemical Diagnosis,* Cambridge, Mass., 1950.
9. *Advances in Clinical Chemistry,* New York (annual publication). Vol. 1 appeared in the summer of 1958.

Two problems will soon engage the attention of the student in studying and reporting scientific papers: (1) selecting the essential points so as to take into account the time available for an oral presentation and to secure appropriate emphasis; (2) dealing as much as possible in terms of *evidence* rather than of *the opinion* of an author.

LIBRARY PROBLEMS

1. Davis showed in about 1942 that the serum proteins bound to various degrees the several sulfonamides then available. If one dialyzes serum against a relatively large volume of water solution containing 10 mg. sulfadiazine per 100 ml., the serum may well reach a level of, say, 13 mg. sulfadiazine per 100 ml. Does this mean that higher, more effective levels of the drug will be reached in the plasma of the treated patient? In a protein-low transudate?

2. A controversy is recorded in the literature as to whether or not the brain must be analyzed for alcohol to discover whether or not a subject was intoxicated; the validity of blood concentrations has been questioned. Find an important paper in this controversy and discuss the question.
3. Case 38052 of the Massachusetts General Hospital (*New England J. Med.*, **246,** 193 [1952]) records the urine protein at 23.7 g. per 100 ml. Decide whether the *concentration* or the *daily excretion* represents the more valuable information. Decide which was actually meant in this case. Similarly, consider whether in case 38041 (*ibid.*, p. 146 [1952]) the paracentesis fluid contained 2.76 g. of protein *in all*, or per 100 ml.
4. From case 42441 (*New England J. Med.*, **255,** 863 [1956]) decide what was meant by this sentence: "The 24-hour urinary excretion of calcium ranged between 3.5 and 8 mg., the phosphorus between 29 and 138 mg., and the uric acid between 13 mg. and 35 mg. per 100 ml."
5. The urea clearance determination contains the fraction U/B, the ratio of the urinary urea concentration to the blood concentration. Is this an exception to the usual principle that in urinalysis we are interested in the total amount of a substance excreted per unit of time and not in the urine concentration?
6. Calculate, using representative values: *total nitrogen*, in milligrams per 100 ml. of blood serum; percentage of this that is *nonprotein nitrogen;* percentage of the total N that is *ammonia nitrogen*. Calculate also the percentage of the total serum halogen that is *"protein-bound" iodine*. Calculate the ratio of average normal serum protein to cerebrospinal protein concentration. Can you estimate readily the percentage of the total serum protein that is alkaline phosphatase? Consider the relative contribution of enzymes to the *liver protein* and to the *serum protein*. Can you calculate the percentage of the total serum steroid that any specific hormone represents?
7. Two commonly used tests of kidney function are the *phenolsulfonphthalein excretion test* and the *urea* or *creatinine clearance determination*. Each depends on estimates of the rate of elimination, in one case of an administered indicator; in the other case, of a normal blood constituent. Both will be inaccurate if there is urinary retention. Consider carefully which test will be the more seriously affected by retention.
8. The plasma prothrombin is measured by determining the *prothrombin time*. What conditions must be met before the time recorded measures the prothrombin? Is the prothrombin level directly related to the time? Inversely? How is it related?
9. Flame photometric analyses (for example, for Na^+ or K^+) have been greatly assisted by the introduction of an *internal lithium standard*. Explain how this is done. Ordinarily one dilutes 1 ml. of serum to 250 ml.

with distilled water plus lithium chloride and feeds the resultant solution through an atomizer into the flame. An analyst adds the correct amount of the lithium solution and then carelessly dilutes the solution to about 260 rather than 250 ml. Will there be an error? Is this a concentration determination?

2

HOW THE HYDROGEN ION DISTRIBUTES ITSELF

Acid-base balance is often found to be a difficult subject. This probably means that some simple underlying principle has not been well understood. The most likely explanation is that we have misunderstood the behavior of *weak acids* and of the *hydrogen ion.* If we are to try here to uncover a missed concept, you must agree to a reconstruction of the subject from the ground up. No amount of discussion of the clinical aspects of acid-base balance can compensate for a missed preliminary idea.

pH. We are concerned in medicine with low hydrogen-ion concentrations. Rather than deal with awkward values like 0.00001 N or $1 \times 10^{-5}N$, we learn to express this as $10^{-5}N$; and furthermore we use the exponent only, without its minus sign, and say that the pH is 5.0. If we do this, we can cover the enormous range from a N H^+ concentration to $0.000\,000\,000\,000\,01N$ H^+ by means of a scale extending from zero to 14.

We pay a considerable price, however, for the simplicity of this scale. It deprives us of a ready sense that a solution of pH 4.7 has twice the hydrogen-ion concentration of one with a pH of 5.0; or that a solution of pH 4.0 has ten times the hydrogen-ion concentration of one at pH 5.0. Here, incidentally, are two good "rules-of-thumb" to keep one from getting lost on the pH scale: Every time the pH drops 0.3 unit, the hydrogen-ion concentration is doubled (0.3 being the log of 2); when the pH drops 1 unit, the $[H^+]$ is increased by ten times. If in addition you are quick to recall, for example, that a pH of 9 means $10^{-9}N$ $[H^+]$, you are equipped to range about on the pH scale without confusion.

Titration. This is the technique we use for discovering the presence of substances in solution that bind hydrogen ion. When we titrate, we purposely vary the hydrogen-ion concentration over a region in which we are interested, to see what H^+-binding we may encounter. But before we try to recognize *binding agents*, we ought to see how the *pure solvent* behaves.

We shall use HCl and NaOH to cause the pH to change. This is almost the same as adding and removing H^+ because, according to modern measurements, HCl and NaOH probably are completely dissociated in water solution; Na^+ and Cl^- do not enter into reaction with H^+ or OH^-.

Suppose we take 50 ml. of distilled water. Perhaps the initial pH is 6.0 (why not 7.0?). We begin to titrate by adding 0.05 ml. (one drop) of *N* HCl. We find the pH has fallen to 3.0. Nothing in the solution has resisted the change in pH; essentially all the hydrogen ions added are still there. (The particle that we shall call the free H^+ is actually hydrated in water solution, but for simplicity here we can ignore this fact without penalty.) The pH is 3.0 because we added 0.05 ml. × 1 (milliequivalent per milliliter) or 0.05 milliequivalent of H^+ (note that the unit of normality is equivalent per liter or milliequivalent per milliliter). This quantity in 50 ml. gives us a concentration of 0.001 equivalent H^+ per liter, which is $10^{-3}N$; hence the pH is 3.0. In the same way a drop of *N* NaOH brings us to pH 11.0. (You may have a little more trouble in calculating why this is the pH reached.)

Here is our domain, pH 3 to 11, for the time being—a very easy region because the quantity of H^+ or OH^- required to change the pH is negligibly small, unless there is something else in the solution. Only in connection with the gastric juice do we need to penetrate the more difficult region outside this range.

Suppose, instead of water, we now titrate acetic acid at a $0.1N$ concentration. Our initial pH for this solution is about 3.0. As we add *N* NaOH from the buret, we find that far more is now required to raise the pH. If we plot the amount needed, we get a curve that looks like Fig. 2.1. For a time the quantity of NaOH required to produce a given pH rise steadily increases; then it begins to decrease, and finally above pH 8.0, our solution again be-

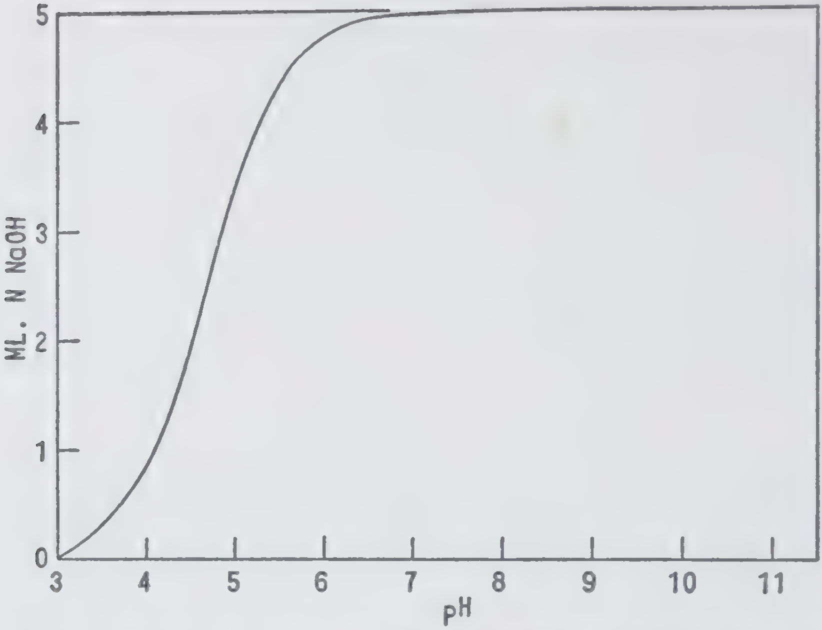

Fig. 2.1 How the pH of a 0.1*N* acetic acid solution changes as *N* NaOH is added. The resistance to pH change is limited to a special part of the pH scale, symmetrically distributed around the *pK′*, 4.7. Above pH 8 the solution is as inert to NaOH addition as pure water. This would also be true below pH 2. The titration curve may also be located by proceeding in the opposite direction, i.e., titrating *acetate ion with HCl.*

haves like pure water. There is nothing present that tends to bind H^+ or OH^- above pH 8.0.

Notice that after we have finished this titration, we can retrace the whole curve by titrating with HCl. The result is the same, beginning at either end; all we need to do is to vary the pH in the range 3 to 8 to discover the presence of the dissociating system $HA \rightleftharpoons A^- + H^+$. In this equation HA is a weak acid, a substance that *releases* H^+. Similarly we may call A^- a weak base because it *binds* H^+; this is the Brønsted terminology. Why is a sodium acetate solution alkaline, and why does its titration consume H^+? This is because the acetate ion is a *base*, a substance that tends to bind H^+.

We can explain the large amount of NaOH required to raise the pH of the acetic acid solution by pointing out that the HA present consumes the added OH^-:

$$HA + OH^- \rightarrow A^- + H_2O \tag{2.1}$$

But why does the curve have its special symmetric shape? We have to turn to the mass-action law to understand this.

For the reaction, $HA \rightleftharpoons H^+ + A^-$, the mass-action law states that at equilibrium (which is reached almost instantly),

$$\frac{[H^+] \times [A^-]}{[HA]} = K$$

That is, H^+ and A^- are formed until their concentrations are large enough to make the backward reaction go as fast as the forward one. The more H^+ and A^- this takes, the larger is K. Therefore K measures the strength of the weak acid. By a weak acid we mean an acid that dissociates only partially. HCl, no matter how dilute, does not belong to this class.

If we solve for $[H^+]$, we have

$$[H^+] = K\frac{[HA]}{[A^-]}$$

Putting this in logarithmic language yields

$$\log [H^+] = \log K + \log \frac{[HA]}{[A^-]}$$

This equation is multiplied by -1 (in a slightly devious manner) to become

$$-\log H^+ = -\log K + \log \frac{[A^-]}{[HA]}$$

If we let pK stand for $-\log K$, just as pH stands for $-\log [H^+]$, we get

$$\mathrm{pH} = pK + \log \frac{[A^-]}{[HA]}$$

This derivation is traced here to remind you that this Henderson-Hasselbalch equation is simply the law of mass action in logarithmic form. No new assumptions have been introduced.

(Beware of using BA for A^-—your attention may be diverted from the real actor, A^-, to an innocent bystander, B^+.)

If K is constant, pK is also constant. Therefore this equation shows how the pH of a solution will change as the proportion of A^- is increased and of HA decreased during a titration by the reaction shown in Eq. (2.1). Suppose, for example, we take a weak acid with a pK of 5.0, and add enough NaOH so that 50% of it will be converted to A^-. Then pH = 5.0 + log 50/50; pH = 5.0 + 0.0 = 5.0. (Note that we do not need to deal in any particular concentration unit as long as we have the correct ratio of the two concentrations.)

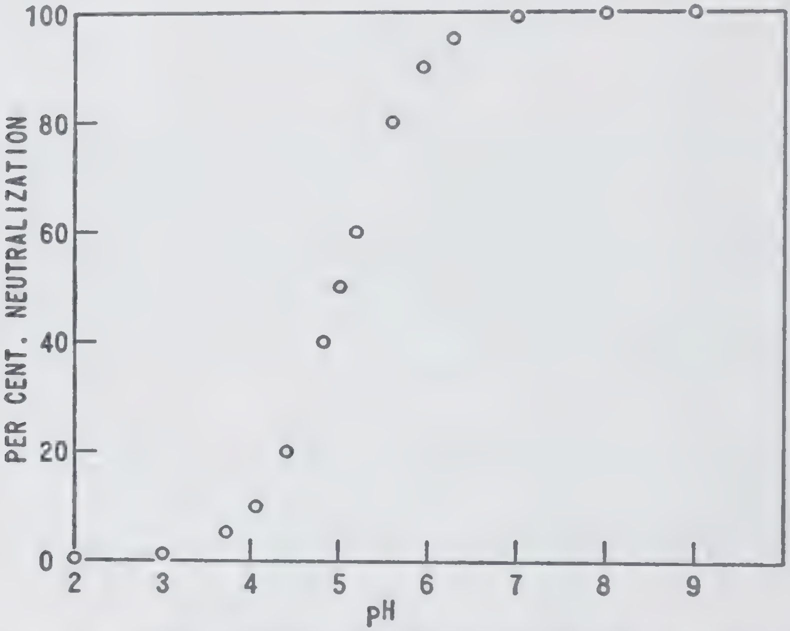

Fig. 2.2 Calculated values for the pH with various degrees of neutralization of a weak acid, using the mass-action law. The sigmoid form actually found is what the mass-action law predicts.

This brings us to the important point that the pK is the point of half-neutralization of a weak acid. Thus we pinpoint the region of buffering of a weak acid by citing its mid-point. If we want a buffer to help us keep the pH at 9.0 we shall choose one whose pK is as near to 9.0 as possible.

Let us place the point thus obtained on our graph (Fig. 2.2) and calculate a few other points. If the quantity of NaOH were large enough to convert 80% of the HA to A^-, the pH would be

$$5.0 + \log \frac{80}{20} = 5.0 + 0.60 = 5.60$$

If, instead, we formed only 20% A^-, the pH would be

$$5.0 + \log \frac{20}{80} = 5.0 - \log \frac{80}{20} = 4.40$$

These two points are added to the graph; they fall symmetrically around the mid-point.

In the same way we get the pH of 90% neutralization as $5.0 + \log 90/10 = 5.95$, and of 10% neutralization, $5.0 - 0.95 = 4.05$; of 99% neutralization as $5.0 + 2.0 = 7.0$; of 1% neutralization as $5.0 - 2.0 = 3.0$; of 99.9% neutralization as $5.0 + 3.0 = 8.0$; of 0.1% neutralization as $5.0 - 3.0 = 2.0$, and so on.

These calculations show us that the sigmoid form of the curve obtained for the titration of acetic acid is required by the mass-action law. One new assumption has been introduced in Fig. 2.2, namely, that the moles of A^- formed will be equal to the equivalents of OH^- added. This is an adequate approximation between pH 3 and 11, where comparatively little OH^- or H^+ is required merely to change the H^+ concentration.

(Outside this range a substantial part of the HCl or NaOH added does not serve to interconvert HA and A^- but merely to change the hydrogen-ion concentration. For example, to carry 50 ml. of water from pH 3.0 to pH 1.0 requires 5 ml. of N HCl. Or to look at it in reverse, a titration of gastric juice from pH 1.0 to 3.0 would involve the titration of almost all the free H^+ present.)

Now visualize the titration of a few other weak acids. First with $0.1M$ H_3PO_4, one discovers a *region of dissociation* centered about pH 2.0, where the reaction is $H_3PO_4 \rightleftharpoons H_2PO_4^- + H^+$, which is mainly terminated at pH 4.5. But then above this pH one encounters the very important *second* region of buffering, centered at about pH 6.8. At this the pK value we have 50% $H_2PO_4^-$ and 50% $HPO_4^=$. (Are H_3PO_4 and $PO_4^{\equiv}$ totally absent?) This titration is terminated below pH 9.0; then, after a short interval devoid of buffering, we finally strike the *third* dissociation, as $HPO_4^=$ becomes $PO_4^{\equiv}$. With a pK of about 12, one would suppose that this dissociation would be of no physiological importance. Nevertheless this third H^+ has to be eliminated in the calcification of bone.

(Can we detect that the three groups we are titrating in this experiment lie in the same molecule rather than representing three different weak acids? Are the three dissociable structures on the molecule, $(HO)_3P{=}O$, inherently different?)

Next we may visualize the titration of NH_4Cl. Upon adding HCl, we discover no structure that will *accept* H^+. But by adding NaOH, we find a substance that will *dissociate* H^+ to give a symmetrical titration curve centered at pH 9.4. What weak acid is present? Clearly it is NH_4^+, and the reaction is

$$NH_4^+ \rightleftharpoons NH_3 + H^+$$

We can fit this system to the Henderson-Hasselbalch equation if we are careful to put the H^+-poor form in the numerator and the H^+-rich form in the denominator as usual; thus

$$\mathrm{pH} = 9.4 + \log \frac{[NH_3]}{[NH_4^+]}$$

Any treatment of this dissociation that requires us first to *hydrate* ammonia and the amines, RNH_2, to NH_4OH or RNH_3OH introduces an artificial dualism. Besides, the extra effort involved in such an indirect approach becomes prohibitive when we come to deal with the dissociation of the substituted ammonium groups of proteins and other complex molecules.

We can arrange some of the important weak acids in a table of *decreasing* strengths:

$$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \qquad pK = 2.0$$

$$HOAc \rightleftharpoons H^+ + OAc^- \qquad pK = 4.7$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad pK = 6.1$$

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{=} \qquad pK = 6.8$$

$$NH_4^+ \rightleftharpoons H^+ + NH_3 \qquad pK = 9.4$$

$$HCO_3^- \rightleftharpoons H^+ + CO_3^{=} \qquad pK = 9.8$$

$$HPO_4^{=} \rightleftharpoons H^+ + PO_4^{\equiv} \qquad pK = 12.0$$

At the same time we obtain a list of conjugate *bases* at the right. These become stronger as we go down the list because their tendencies to associate H^+ becomes stronger. In these lists we have included anions, cations, and neutral molecules. But if we insist on dealing with them in different classes according to their charge (i.e., by treating H_2CO_3 but not NH_4^+ as an acid), we introduce artificial complexities.

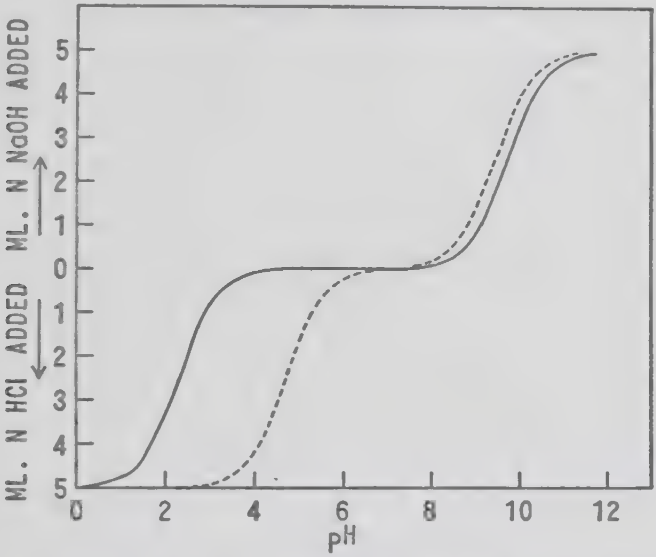

Fig. 2.3 Titration curves for ammonium acetate solution (dotted curve) and for an amino acid glycine (solid curve). The titrations were started at the middle, represented by crystalline ammonium acetate or glycine, but they might quite as well have begun at either end. Note the similarity of the behavior of the two.

Suppose now that we were to titrate a solution of ammonium acetate. With NaOH we should discover the NH_4^+ dissociation, as for NH_4Cl. With HCl in the other direction we should note the uptake of H^+ by the acetate ion. The whole titration curve of this solution would show two buffering regions, just as H_3PO_4 shows three (Fig. 2.3). Now suppose we join the two radicals of ammonium acetate into a single particle by making a carbon-to-nitrogen bond

$$H_3{}^+NCH_2COO^-$$

What we have written is the glycine molecule in the *correct* form, that is, the overwhelmingly predominant form. (To write it as NH_2CH_2COOH is equivalent to designating ammonium acetate as $(NH_3)(CH_3COOH)$, when we know that the hydrogen ion associates itself much more strongly to the nitrogen atom. The dissociation of glycine becomes extremely confusing if we make the mistake of beginning with the second structure.)

Now if we titrate glycine with NaOH and HCl, we get a result quite similar to that for ammonium acetate (Fig. 2.3) except for characteristic shifts in the positions of the two buffer regions. Adding NaOH pulls the H^+ off the charged amino group ($pK' = 9.7$) to form $H_2NCH_2COO^-$; adding HCl adds a H^+ to the carboxylate group to form $H_3{}^+NCH_2COOH$ ($pK' = 2.3$).

Glycine is unduly simple as a model of *protein* titration behavior, but it is useful to remember that for both, beginning at their isoelectric points, adding H^+ eliminates charged carboxylate groups so that cationic nitrogenous groups predominate, whereas adding OH^- groups eliminates the plus charges on these nitrogenous groups so that anionic groups predominate.

Calculating the pH of a Buffer System. We need to distinguish two different situations in such calculations. First we can prepare a buffer by mixing 10 ml. of $0.1N$ weak acid, $pK = 5.0$, with 5 ml. of a $0.1N$ solution of the salt of the weak acid. This combination yields a ratio $[A^-]/[HA] = 5/10$, and therefore $pH = 5.0 + \log 5/10 = 4.7$.

But suppose, instead, we add 5 ml. of $0.1N$ NaOH to the 10 ml. of $0.1N$ weak acid. Now the fraction $[A^-]/[HA]$ will have the value 5/5, and $pH = 5.0 + \log 1 = 5.0$.

This illustration shows us that we must take into account any chemical reactions occurring before applying the Henderson-Hasselbalch equation.

Are *pK*'s Constant? So far we have assumed that the pH of a buffer depends *entirely* upon the ratio of $[A^-]$ to $[HA]$. This implies that we could dilute a buffer to any extent whatever without pH change. In actual practice discretion is necessary. Because of nonideal behavior as a solution becomes more concentrated in ions (ionic strength), all the ions present move less effectively, and the solution behaves as though it contained smaller levels of each ion than are actually present. We may correct each ion concentration specifically for this effect, but instead we prefer in this case to correct the pK, that is, to determine a new apparent pK for the more concentrated solution. For highly accurate work we need to know the pK applying at the ionic strength of our measurement. Such a working pK is designated the pK'.

Knowing the pH Is Not Enough. Simply measuring the pH of a biological solution gives us a picture of very limited value. Usually we like also to know how well the pH is anchored, i.e., how well the solution is buffered. This is not a constant feature but varies from one pH to another. We *titrate* to discover how much NaOH or HCl is required to change the pH at each point. A typical biological fluid contains a variety of buffer systems, and its titration curve shows their overlapping activities.

We may represent an unbuffered solution by a very slender standpipe that is supposed to serve as a hydrostatic reservoir for a city. Only a small amount of H^+ needs to be removed or added to change the level greatly. Introducing a buffer system is like inserting a large bulb in the standpipe at one particular level. Now we get good "buffering" of the water level near the selected level but only in the region of that level. The detailed conformation of the "reservoir" depends upon what buffers are present and at what concentration. By titration we can discover the distribution of the buffering of a biological solution, like blood or saliva, over the pH scale.

Why Does the pH Change with Changes in HA or A^- Concentration? If one is asked why the serum pH rises when the bicarbonate concentration is increased, he may be tempted to say, "because the Henderson-Hasselbalch equation requires it," or to mutter something about a common-ion effect. The fact is that the bicarbonate ion *is a base,* i.e., a substance that binds H^+ with a particular affinity. *Naturally it raises the pH.* If we double the carbonic acid concentration of a carbonic acid-bicarbonate buffer without changing the HCO_3^- level, naturally we also double the H^+ concentration (or lower the pH by 0.3). If we double the HCO_3^- level without changing the H_2CO_3 concentration, naturally we halve the H^+ concentration (or raise the pH by 0.3). Perhaps it is because we try to use the mass-action law in its logarithmic form that we often fail to see this.

Distribution of Hydrogen Ion Among H^+ Acceptors. Suppose we prepare a carbonic acid-bicarbonate buffer, pH = 7.0, and mix it with a $H_2PO_4^-/HPO_4^=$ buffer, pH = 7.4. Something must yield. The solution can have only one hydrogen-ion concentration. Obviously the new pH will lie between the other two. Because of the lowered H^+ concentration it encounters, the H_2CO_3 will release some H^+. The $HPO_4^=$ in turn has been transferred to a higher H^+ concentration and will bind some H^+.

Suppose the new pH is 7.1. Using the *pK* applying to the first system, we calculate

$$7.1 = 6.1 + \log \frac{[HCO_3^-]}{[H_2CO_3]}$$

$$\log \frac{[HCO_3^-]}{[H_2CO_3]} = 1; \quad \frac{[HCO_3^-]}{[H_2CO_3]} = 10$$

For the phosphate system

$$7.1 = 6.8 + \log \frac{[HPO_4^=]}{[H_2PO_4^-]}$$

$$\log \frac{[HPO_4^=]}{H_2PO_4^-]} = 0.3; \quad \frac{[HPO_4^=]}{[H_2PO_4^-]} = 2$$

The H^+ has assumed a distribution between the $2H^+$ acceptors, $HPO_4^=$ and HCO_3^-, which is governed by their relative affinities for H^+ and the supply of H^+ available. What factors determine the pH actually obtained on mixing two such buffers?

If we happened in this case to choose the bicarbonate-carbonic acid system for reference, we may decide that the pH had been established solely by the ratio of the bicarbonate to the carbonic acid concentration. Or we could attribute the pH to the value of the ratio $HPO_4^=/H_2PO_4^-$. This example illustrates that we can use any convenient buffer system as an *indicator* of the pH (even a colored one like phenol red) although that particular system may or may not play a dominant role in *establishing* the pH.

pH Determination. This, of course, is the principle upon which indicators work: The hue changes with the change in the proportions of indicator in the HA and A^- forms. Because we choose deeply colored substances, only traces too small to modify the pH need be added. Consider what determines where the useful range of an indicator lies and how wide the range is.

Very reliable pH determinations are obtained by preparing standard buffers whose pH is well established, giving indicator hues close enough to the unknown to permit close interpolation. A glass electrode, sufficiently permeable to the hydrogen ion but not to other ions, will yield the same potential for solutions of the same hydrogen-ion concentration. In this case extrapolation can be carried further because of the known relationship between electromotive force and ion concentration.

In biological work we must remain alert to the very large effect of temperature upon pH. The normal plasma pH is nearer 7.6 than 7.4 at room temperature. Ideally, observations should be made at a constant temperature close to that prevailing *in vivo*.

Distribution of Hydrogen Ion between Cells and Extracellular Fluid. When the organism is exposed to a certain load of extra hydrogen ions, a portion of these enter the cells and are taken up by hydrogen-ion acceptors there. The importance of measuring the acid-base situation in the blood rests on the premise that the result reflects the situation in the other compartments, which has generally seemed to be the case. Certainly the results do apply to the interstitial compartment as well as to the plasma. If, however, the pH of a bicarbonate-carbonic acid buffer in which muscle is suspended is varied over a wide range by changing the bicarbonate concentration, the bicarbonate ion fails to penetrate so that the muscle bicarbonate level scarcely changes, thereby indicating a nearly constant pH within the muscle (Wallace and Hastings). Furthermore an apparent dissociation of the extracellular pH from the cellular pH has now been proposed in potassium deficiency, with the extracellular phase becoming alkaline while the cellular phase becomes acid. This question will be discussed later. Until the possibility of such uneven distribution of the H^+ is evaluated, the over-all significance of acid-base changes in the blood in any new situation will remain somewhat uncertain.

LIBRARY PROBLEMS

1. The serum choline esterase activity is often measured in liver disease. One method is to let the enzyme split acetylcholine in a buffer solution at pH 8 and then to note how much the pH is decreased by the H^+ released by the reaction. The results are stated in delta-pH units; for example, if the pH falls by 0.28 units, the patient is stated to have 0.28 delta-pH units of choline esterase. Is this a concentration unit? Does a patient with 0.56 delta-pH units have twice as much serum choline esterase as one with 0.28 units? Would this method of observing cleavage work at pH 3.0, for example, if this were the pH optimum of the enzyme?
2. In the following reaction, would a drop in pH occur which could be used to measure the extent of the reaction?

   A cleavage of glucose-6-phosphate (pK's 2.0, 6.5) to glucose and inorganic phosphate (pK's 2.0, 6.8, 12.4) at pH 4.5; at pH 7.4.

   What do you think of the likelihood that such phosphoric esters would have an acidifying effect when ingested in the diet? Check two or more textbook discussions of the acidifying effect of a protein diet to see whether they ascribe part of the effect to the presence of phosphate in protein.
3. Describe carefully what would happen if one mixed a $0.1M$ acetate buffer, pH 4.7, with an equal volume of a $0.1M$ NH_4^+–NH_3 buffer, pH 9.4. Would the resultant solution be well buffered?
4. The titratable acidity of urine is the number of milliequivalents of NaOH required to titrate a 24-hr. urine sample (using an aliquot portion, suitably diluted) from its original pH to pH 7.4. The value obtained is considered to equal the number of milliequivalents of H^+ that have been introduced into the urine by renal activity. As much as 50 milliequivalents of titratable acidity per day may be found.

   a. Would not a determination of the pH of the well-mixed 24-hr. urine do as well? Why?

      Gastric juice, on the other hand, is titrated from its natural pH to a pH of 3.3 to measure the *free acidity*, i.e., the concentration of *free* hydrogen ions.

   b. Would not a determination of the pH of the same sample of gastric juice serve as well? Why?

   c. If the answers for parts (a) and (b) are different, be sure your discussion explains the difference.

5. Gastric juice is usually diluted 1:10 before titration, and the titration is terminated at pH 3.3, using Toepfer's indicator. Calculate what percentage of the free H^+ will have been titrated at this pH if the gastric juice had an original pH of 1.0. What is meant by a tubeless gastric analysis?
6. In an experimental study a student is advised for convenience to dilute a series of 24-hr. urine samples to 1000 ml. before analysis. He argues that this will change the pH of the samples and hence that pH determinations should be made before adding water. Discuss his objection carefully.
7. In the bromsulfalein test of liver function, a specified dose of the indicator *bromsulfalein* ($pK = 8.8$) is injected, and after a given interval blood is drawn and a serum sample is analyzed colorimetrically for the indicator. A normal person practically clears his blood of the material in 30 min.

   An analyst proceeds as follows: He prepares a bromsulfalein standard solution. A photometer, fitted with a suitable filter, is adjusted to zero optical density (or 100% transmittance) with a water-filled cuvette in the well. He then determines the optical density of the standard and of the unknown serum, and from the relation between the two, he calculates the concentration of the unknown serum.

   There are two or three distinct errors in the analytical part of the procedure as described. Discuss how you would carefully avoid these.

   Does the presence of jaundice interfere with the execution of the foregoing test?
8. The dissociating groups of proteins are contributed mainly by the *side-chain* groups of the amino acids and usually only at the end of a polypeptide chain by *α-amino* and *α-carboxyl* groups. For a given protein determine from a textbook the analytical results for the amino acids that will contribute dissociating groups. Represent the protein molecule diagrammatically by a circle, and attach to it the correct number of dissociated and undisassociated groups to represent the condition at the isoelectric point. Show how you would convert it into (a) a form that would move toward the cathode, and (b) to a form that would move toward the anode, upon electrophoresis. What factors determine how far such molecules move in the electric field in a given time interval?
9. Suppose that you wanted to administer substantial amounts of glutamic acid or sodium glutamate, as has been recommended in hepatic coma. Trace a rough titration curve for glutamic acid, using *pK*'s of 2.2, 4.3, and 9.7. From the curve decide what pH values would be characteristic of a glutamic acid solution; of sodium glutamate. Can any glutamate solution be devised which is without effect on the neutrality, taking into account (a) the immediate effect on the neutral-

ity, (b) the effects on neutrality upon complete catabolism? This problem was encountered in case 41432, *New England J. Med.*, **253,** 737 (1955), which should be consulted.

10. Find and report a paper discussing the distribution of injected hydrogen ions between cells and extracellular buffers.
11. Organic acids are determined in urine by a titration method. Look up this method and determine whether the titration will include the amino acids lost into the urine.
12. How is a metal ion (for example, Mn^{++}) distributed among a group of chelating substances present in a single solution? Is this behavior similar to that of the distribution of H^+ among buffer anions?
13. Occasionally the pH of the gastric juice has been measured at intervals and the pH values averaged to produce a mean pH. Is this a justifiable calculation?

3

THE DISTRIBUTION OF SODIUM AND CHLORIDE

Water Transfer. Before considering the distribution of these abundant ions, perhaps we should stop to think about water distribution. A preliminary chapter on that subject is not practical here because we can hardly extricate the subject from ion distribution.

Most investigators believe that the cells of higher organisms mostly behave passively to water passage; i.e., movement of water by free diffusion is fast enough to hide any directed movements of water. This does not mean that living cells cannot "concentrate" water, which certainly occurs in renal activity and in the formation of saliva and some other secretions. The urine may be either hypotonic or hypertonic to the plasma.

Increases in water concentration may conceivably be produced in either of two ways: (1) by sequestering a portion of extracellular fluid and then transferring solute molecules from it through a barrier that at the same time retards the movement of water molecules; (2) by taking hold of the water molecules one by one and transferring them into a separate compartment through a membrane that retards the movement of solute molecules. The former appears the more practical because only a comparatively small number of molecules needs to be moved. That is, if water *per se* is to be transferred, there are about 55 mol. of water for each liter, or roughly 200 times as many molecules to be moved as there are of all solute molecules combined. True, the concentration gradient against which water must be moved is relatively less steep (for example, from $55.3M$ to $55.4M$), but each transfer is likely to involve considerable inefficiency, thus adding up to a much greater energy expenditure.

The only diagnostic determinations that are measures of water concentration are the determinations of osmotic pressure, or freezing-point lowering; for example, of urine. These may represent explorations of the distribution of water *per se,* i.e., the behavior of the kidney in concentrating water or solutes. In practice, the specific gravity is more frequently measured for the same purpose. Now, the presence of most solutes does *increase* the specific gravity of aqueous solutions, but some instead *decrease* the gravity. Furthermore the increase in density does not really measure the *number* of molecules of solute present. Only because various urine samples are likely to contain a rather similar collection of solutes are we able to estimate the extent of urine "concentration" by measuring its specific gravity. The presence of abnormal solute patterns, as in glucosuria or proteinuria, may upset completely the interpretation of this measurement.

Although we occasionally measure the freezing point of plasma or serum, the investigation of the same property of the cellular fluids is much more difficult and equivocal. Hence there is no routine diagnostic investigation of the partition of water between cellular and extracellular fluids. What does receive tremendous interest (to draw a fine distinction) is the distribution of *fluid volume* between these two compartments of the body. This, however, is principally the story of the distribution of abundant solutes like Na^+, Cl^- and K^+, with the water apparently playing a passive role, moving freely to produce equality of osmotic pressure. Therefore we shall pass on to discuss ion distribution.

Law of Electroneutrality. A first simplifying chemical principle is that no macroscopic quantity of a fluid will contain analytically more positive charges on its ions than negative charges. If we find that a salt solution contains $0.2M$ Na^+ and $0.1M$ Cl^-, we know that we have another anion to find. Of course, if we record our analysis as 460 mg. % Na^+ and 355 mg. % Cl^-, we may remain unaware of the discrepancy. We must deal in units of charge of the ions, i.e., in *milliequivalents per liter* (millinormality) to make use of this important simplifying principle. If we want to convert an analytical result from milligrams per 100 ml. to the more valuable unit, we multiply by 10 (to get milligrams per liter) and divide by the equivalent weight (for example, 23 for Na^+, 20 for Ca^{++}). The conversion in the other direction perhaps deserves no attention if we regard milligrams per 100 ml. as an obsolescent unit for ion concentrations.

The Extracellular Fluid. For serum the Law of Electroneutrality may be stated as follows:

$$\underset{142}{[\mathrm{Na}^+]} + \underset{5}{[\mathrm{K}^+]} + \underset{5}{[\mathrm{Ca}^{++}]} + \underset{3}{[\mathrm{Mg}^{++}]}$$

$$= \underset{103}{[\mathrm{Cl}^-]} + \underset{27}{[\mathrm{HCO_3}^-]} + \underset{17}{[\text{prot.}^-]} + \underset{5}{[\text{other org. anions}^-]}$$

$$+ \underbrace{[\mathrm{HPO_4}^=] + [\mathrm{H_2PO_4}^-]}_{2} + \underset{1}{[\mathrm{SO_4}^=]}$$

Here we include only relatively abundant ions, neglecting a great many trace metals and lumping many trace anions together. Again taking advantage of the Law of Electroneutrality, we can represent the electrolyte picture by two bars of equal length, one to represent anions, one to represent cations (Fig. 3.1).

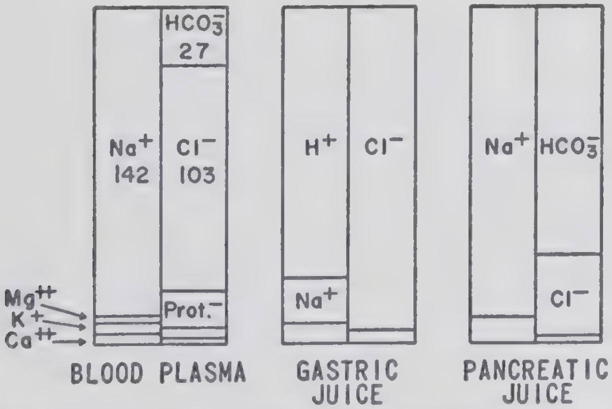

Fig. 3.1 Bar diagrams to illustrate electrolyte composition of human serum, gastric juice, and pancreatic juice. In the first diagram the trace anions are proteins, other organic anions, phosphate, and sulfate. The trace ions are not diagrammed in detail in the other sketches.

This picture is referred to as the *electrolyte framework* of the extracellular fluid. The term is intended to convey the essentiality of the extracellular electrolytes—without the ions the fluid cannot be formed or retained. Three ions, Na^+, Cl^-, and HCO_3^- predominate in this picture; a convenient approximation is tentatively to ignore changes in the other constituents and to treat the lower 12 or 13 milliequivalents of the cation bar and the lower 25 odd milliequivalents of the anion bar as fixed "pedestals." Changes in $[Na^+]$, $[Cl^-]$, and $[HCO_3^-]$ are much more likely to account for changes in the height of the bars, $[Na^+]$ being the best single measure of the height of the electrolyte framework.

The sodium ion is a uniquely necessary cation in the extracellular fluid, just as potassium ion is in the cells. We could suppose that these two similar ions would contribute relatively inert, indistinguishable cationic and osmotic properties, but in fact very little replacement of the extracellular Na^+ by K^+ is tolerated. Such substitution is marked by changes of electric potential and irritability of muscle and nerve and (critically) by arrest of the heart muscle.

The choice of serum as a typical extracellular fluid may be criticized, principally because the protein content is higher than that of interstitial fluids from which it is separated by the rather permeable capillary walls. The behavior we anticipate with regard to an inert membrane is that the concentration of solutes in mols *per kilogram of solvent* (not necessarily in mols *per liter of solution*) will be the same on both sides. From a strict chemical point of view, therefore, we perhaps should correct these concentrations to milliequivalents per kilogram of water by dividing by 0.93, there being about 93% water in serum.

Another source of small differences in the ion concentrations of plasma and interstitial fluid is the *Gibbs-Donnan effect*, which is illustrated in a highly diagrammatic fashion in Fig. 3.2. Suppose we begin with NaCl distributed evenly between the two compartments, represented diagrammatically by five sodium and five chloride ions on each side, the separating membrane being easily permeated by water, Na^+ and Cl^-. Now let us add to the right-hand chamber a quantity of sodium proteinate or of sodium ribonucleate. The organic anion is too large to pass through the pores of the membrane. There is now a strong tendency for Na^+ to pass to the left to equalize the two Na^+ levels. For this to occur, Cl^- must also move to the left *against the concentration gradient.* If this shift continues until the Na^+ is uniformly distributed, a Cl^- gradient as large as the original Na^+ gradient will be created.

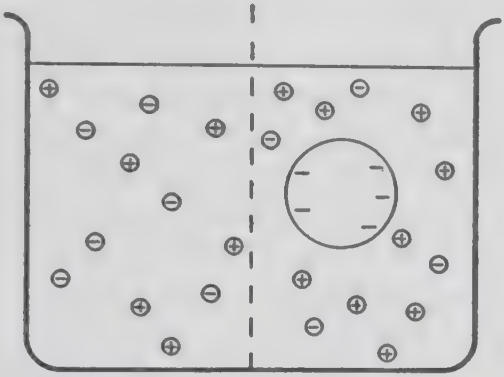

Fig. 3.2 Diagrammatic representation of the Gibbs-Donnan equilibrium. The sodium salt of a nondiffusible multi-valent anion added to one compartment of an NaCl solution has caused a redistribution of Na and Cl ion, with Na^+ moving to the left, accompanied by an equivalent migration of Cl^-. Under these conditions Cl^- is moving against a concentration gradient. At equilibrium there are 12 particles on the left, 14 on the right; hence the osmotic pressure difference is greater than that created by the macromolecule *per se.* This effect will increase if the macromolecule gains more minus charges, but it will disappear if it becomes isoelectric.

Obviously this will not happen, but instead, a compromise will be reached whereby net movement of the ions stops when the tendency of the Na^+ to continue to pass to the left is exactly opposed by the tendency of the Cl^- to pass to the right. This situation is represented by equal ratios of the two ion concentrations in opposite directions between the two phases, i.e.,

$$\frac{Na_1{}^+}{Na_2{}^+} = \frac{Cl_2{}^-}{Cl_1{}^-}$$

Thus, in the illustration, 9 is to 6 as 6 is to 4. But if we consider Cl^- only in this example, we see that it has moved *against* a concentration gradient.

Note that as an incidental consequence, the total number of extra particles on the right-hand side is even greater because of the Donnan effect than it would be with the presence alone of the colloidal particle. It is for this reason that the colloid-osmotic pressure of the plasma rises with the pH as the proteins dissociate H^+ and gain more negative charges per molecule. The osmotic pressure due to the proteins falls to a minimum at an average isoelectric point where the proteins have no net charge.

The chloride concentration of the red blood cells, expressed in milliequivalents per kilogram of water, is about seven-tenths that of the plasma water, although the cell appears to be readily permeable to Cl^-. This is undoubtedly a Donnan effect arising from the much higher protein concentration of the red cells.

Volume Partition between the Plasma and Interstitial Fluid. The plasma proteins are reintroduced into the circulation by lymphatic activity faster than they escape from it, until their level is higher in plasma and a substantial colloid-osmotic pressure is set up. Accordingly fluid tends constantly to enter the circulation, but this tendency is opposed by the greater average hydrostatic pressure of the bloodstream.

In this control of volume distribution, the albumins are far more important, gram for gram, than the other plasma proteins because there are several times as many molecules in a gram of albumin as in a gram of one or another of the globulins. If albumin entry into the circulation falls behind its escape from the circulation, either the plasma volume may not be maintained or the extravascular phase may be abnormally large, depending upon other circumstances.

The Digestive Juices. These are also *electrolyte solutions.* The fluid that you may see being aspirated from the stomach or small intestine of a patient after surgery may not look very valuable, but we need to remember that, liter for liter, it resembles extracellular fluid in electrolyte concentration. Figure 3.1 shows the composition of two of these juices. In gastric juice, H^+ tends to take the place of part of the Na^+, and the bicarbonate ion is undetectable. (Why should this be the case?) The pancreatic juice in contrast is an alkaline juice, differing mainly from the extracellular fluid in its high bicarbonate concentration. (Does this necessarily imply that HCO_3^- is concentrated by the gland?) Bile and intestinal juice are somewhat intermediate in ionic composition between the pancreatic juice and the extracellular fluid.

The volumes secreted daily are large. Classical estimates place the daily saliva volume at 1500 ml., the gastric juice at 2500 ml., the bile at 500 ml., the pancreatic juice at 700 ml., and the intestinal juices at 3000 ml. per day. If we take 7 to 10 l. per day as a typical total, we have roughly 60% of the extracellular solution (and of the electrolytes!) secreted into the alimentary canal every day. For a child or an infant the turnover is even faster. True, the saliva is a hypotonic solution, so that its volume should be discounted, but the other juices are practically isotonic with the extracellular fluid.

These considerations make digestion, or even the maintenance of the alimentary canal, a hazardous operation. Of course no such volumes are present in the digestive tract at any one time; instead, resorption occurs constantly to produce an "internal circulation of the salts."

Nevertheless this is a vulnerable point in our body function because so many diseases interrupt the absorption of these fluids. Diarrhea and vomiting may arise from digestive diseases; vomiting in particular may be associated with almost any illness. Unfortunately these disturbances are also generally associated with interrupted food intake. The resulting situation has been termed *acute nutritional failure*. Sodium and chloride are nutritional essentials, but for the person who is eating there is little likelihood of inadequate intake of either, *except on therapeutic regimes or when such unusual losses occur.*

Dehydration. The consequence of the excessive loss of gastrointestinal juices or of extracellular electrolyte by other routes (for example, excessive sweating) is a decrease of the *extracellular volume,* termed *dehydration.* (This definition of dehydration should not be inferred to mean that *only* the extracellular compartment is affected.) The term *dehydration* is unfortunate in this application because clinical dehydration usually arises as a deficiency of the *electrolyte framework* rather than of the *water,* although either origin is possible. Application of the intuitive notion that more water ought to correct dehydration is likely to have the opposite effect: If given water by mouth the patient may shortly vomit, losing more electrolyte with the water; even if it is given by vein (for example, as 5% glucose) the water is likely not to be retained but instead to appear in the urine. Here it will be accompanied by a little additional salt, so that the situation is only made worse. On the other hand should the kidney not respond in this way, and the water be retained without the electrolyte framework, *water intoxication* is likely to be produced, the patient becoming disoriented as the central nervous cells imbibe water from the abnormally dilute environment.

The mental trap presented by the term *dehydration* is shown by this paradox: If *water* (for example, as 5% glucose) is infused into a fasting subject at a rate of about 6 l. per day, he will soon become *dehydrated* (Schemm; Stewart and Rourke). The kidneys become less efficient in resorbing salt when the urine volume is increased greatly, so that a net loss of electrolyte occurs.

A person may lose a one-fourth or one-third of his extracellular fluid before dehydration is readily detected on examination, although weakness would be evident and weight loss could be observed. In clinical dehydration over one-half of the extracellular fluid volume may be lost. The *plasma volume* can scarcely be maintained in such a deficiency. Two consecutive events intervene as the volume decrease continues: The renal circulation shuts down to a level where the efficiency of the kidney in neutrality regulation is lost (acidosis then contributing to the picture) and terminal *shock* or circulatory collapse occurs. The effectiveness of replacement by saline solutions in severe dehydration is dramatic.

Dehydration may also be caused by a primary deficiency of water rather than of electrolyte, but this is far less common. This origin is encountered in primary thirst, however imposed, and in certain patients (usually with central nervous system injuries) whose thirst receptors are not responding normally to the hyperosmolarity of the body fluids. The water intake of the patient exposed to unusual losses (sweating, fever, diabetes insipidus) requires careful attention when the patient is unaware of his own needs.

Urine Formation. Secretion of the urine plays a decisive part in maintaining the concentration as well as the neutrality of the extracellular fluid. The renal adjustments of Na^+ and Cl^- conservation are decisive in volume control because of the predominance of these ions in the extracellular fluid. The Na^+ economy receives the greatest emphasis.

Present conceptions of tubular behavior may be summarized briefly as follows:

In the *proximal* tubule 30% to 80% of the inorganic ions filtered through the glomerulus are transported back into the bloodstream. This transport apparently occurs without the development of significant concentration gradients because a proportional quantity of water readily follows the ions by osmosis. A number of excretion products are, however, concentrated in the tubular urine by this process, and furthermore, glucose and amino acids are reabsorbed and the test substance *p*-aminohippurate is secreted into the urine in this segment. Sodium pumping may explain most of the volume loss, with chloride and bicarbonate following passively. The process is held to be nearly isohydric.

In the *distal* tubule hydrogen ion may be concentrated by as much as 800 times in its transfer into the urine. Sodium and chloride may be concentrated by 50 times in the reverse direction in forming dilute urines. A large part of the sodium pumping appears to occur by a mechanism in which K^+ and H^+ compete, possibly to replace urinary Na^+. This becomes the chief locus of K^+ excretion. Another portion of the Na^+ pumping appears to be unlinked with K^+ or H^+ transfer.

Sodium resorption is believed also to occur in the loop of Henle, but the water transfer apparently does not keep pace, and a hypertonic solution is found in the medullary tissue and the papillae, whereas the urine is believed to be hypotonic as it leaves the loop of Henle (Wirz; Berliner). The lag in water transfer is not completely corrected in the distal tubule but may continue into the collecting ducts.

The *anti-diuretic hormone* is necessary for concentration of the urine and may function by making the distal tubule more permeable to water to permit greater osmotic return of water to the surrounding tissue. The average person can concentrate urine to about a 1.4 osmolar concentration. An average daily output of 1200 milliosmoles of solutes has been found, urea accounting for almost one-half of this figure.

A system of self-regulatory mechanisms for the concentration and volume of the extracellular fluid includes (1) the responsivity of the kidney itself to the ion concentrations, (2) release of *anti-diuretic hormone* in response to increased hypertonicity acting upon osmoreceptors, and (3) release of *aldosterone* and possibly similar hormones in response to Na^+ deprivation (perhaps more specifically to hypovolemia). The latter hormones facilitate the reabsorption of Na^+ and the excretion of K^+. These mechanisms tend to keep the electrolyte framework of the extracellular fluid comparatively constant.

The failure of one of these controls is seen in *Addison's disease*, where adrenal cortical tissue has been largely destroyed or is atrophic and Na^+ resorption by the renal tubules is handicapped. K^+ excretion at the same time is abnormally low. The Addisonian patient is in constant danger of dehydration and consequent shock. The serum sodium concentration tends to sink, reflecting an accompanying severe depletion of the *volume* as well as the concentration of the extracellular fluid. The serum K^+ tends to rise above normal levels.

Diabetes insipidus represents the failure of the anti-diuretic mechanism and may arise from hypothalamic or posterior pituitary injury. (What does *diabetes insipidus* mean?)

The ability of the kidney to excrete sodium ion may be handicapped in cardiac disease by a decreased cardiac output, leading to decreased renal blood flow. Since fluid is retained along with the electrolyte, edema results. Difficulties in sodium excretion occur also in cirrhosis and in toxemias of pregnancy. Restriction of intake through the so-called *sodium-free* diets (which obviously must not be continuously sodium-*free*, since Na^+ is nutritionally essential) is used in an attempt to balance the slowed excretion.

Edema is frequently treated by withdrawal of body Na^+ by diuretics and occasionally by feeding an ion-exchange resin. This consists of a granular polymeric carboxylic acid, which binds Na^+

in exchange for its hydrogen ions. Usually a portion of the resin is administered as the potassium salt to avoid K^+ depletion. Such resins must be used only intermittently because of their acidifying action. Mixed anion and cation exchangers can avoid this effect.

We have seen that we secrete a major fraction of our extracellular electrolytes into the alimentary canal daily. The whole volume of the extracellular fluid passes through the glomerulus, however, about every two hours! The hazards of heavy losses are not correspondingly greater, but the delicacy with which the various ions must be conserved or not conserved by tubular processes should be evident. The symptoms of failure of renal adjustments probably arise more from *failure* of appropriate conservation than from *retention* of toxic waste products.

Diagnostic Analysis for Na^+ and Cl^-. These ions are measured in serum mainly in two connections: to investigate the total height of the electrolyte framework (which the Na determination accomplishes adequately), and to obtain information related to the acid-base balance. The first purpose concerns the state of hydration and the operation of the mechanisms (for example, endocrine) that control fluid homeostasis. The second purpose is accomplished by neither analysis alone, nor do the two together do much better.

You will be assisted in your thinking about these analyses if you visualize the part of the electrolyte bar-diagram, which your laboratory analyses permit you to draw, as illustrated in Fig. 3.3. If you learn that the serum Na^+ is 137 and the Cl^- is 115 milliequivalents per liter, you can put these findings together, tentatively assuming that the various smaller components of the "pedestal" are at normal levels. The resulting diagram will show an interval of 10 milliequivalents, which *might* be bicarbonate ion. Hence these analyses together provide *crude evidence* related to the acid-base balance, but quite possibly three-fourths of this 10-milliequivalent interval actually represents ketone anions! Or in a hypoproteinemia perhaps the interval is really 18 milliequivalents.

The chloride analysis by itself seems to accomplish very little. If it is *very high* or *very low*, it may set presumptive upper or lower limits on the bicarbonate level, if we assume limits within which the serum Na^+ *must* lie. The picture usually is too fragmentary, however. An exception may occur in the diagnosis for example of pyloric stenosis, where the chloride is consistently driven in one direction, downward. In most situations the situation is more complex. As for the primitive reasoning, "low serum chloride, therefore always inject saline," let us be reminded that an electrolyte picture that includes a *high* serum chloride (for example, in infantile diarrhea) can be treated by injecting sodium chloride solution, although the injection of sodium lactate is probably superior.

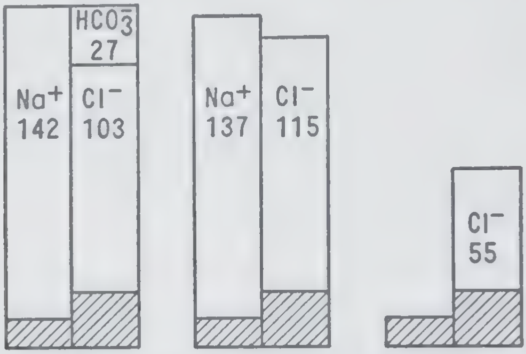

Fig. 3.3 Proposed utilization of fragmentary data. On the left is shown a bar diagram for a normal serum. In the middle case a serum [Na+] of 137 and [Cl−] of 115 milliequivalents per liter have been found. Tentatively it is assumed that no change has occurred in the level of minor components, so that the pedestal is used unchanged. The height of the electrolyte framework is thus indicated with sufficient accuracy. A gap of 10 milliequivalents per liter for the bicarbonate ion appears. The most serious uncertainties in this estimate are (1) that a ketone acid accumulation has occurred so that [HCO_3^-] is overestimated; (2) that a hypoproteinemia has caused an underestimate of the [HCO_3^-]. In the third diagram only a serum chloride was obtained (a case of diabetic acidosis). Here our knowledge of the electrolyte framework is essentially nil.

When the serum Na^+ is measured to investigate the supply of extracellular electrolyte, we may discover a deficiency of perhaps as much as 10 or 15 milliequivalents in its concentration, but this gives us no *direct* measure of the total sodium *content* of the extracellular fluid. If we could measure the total volume of that fluid, the picture would be much clearer. This volume can be estimated by injecting a substance that distributes itself rapidly throughout the extracellular compartment but enters no other compartment. If we measure its level in the serum after a suitable interval for mixing, we can calculate the apparent volume through which it is distributed. In practice we may also correct for the amount that meanwhile has been excreted. The thiocyanate or bromide ions have been used for this purpose. The method has little diagnostic use.

Although hyponatremia usually arises from *handicaps* to the renal regulatory mechanism (dehydration or water intoxication), there is a heterogeneous group of patients whose regulatory mechanisms seem to have become adjusted *to maintain hyponatremia.* Attempts to return their plasma Na^+ level to normal levels are only briefly and difficultly successful and may produce edema. This condition is seen, for example, in very sick patients subsequent to extensive surgery, and it has been referred to as "symptomless" or "sick-cell" hyponatremia. One possible origin is a sustained inappropriate secretion of the anti-diuretic hormone.

The foregoing use of the serum sodium level to evaluate dehydration breaks down entirely, of course, when water deficiency (rather than electrolyte deficiency) is a primary contributor to the loss of the extracellular fluid volume. The consequence of pure water deficiency is of course *hypernatremia.*

Interpretation of diagnostic analyses for these two ions, Na^+ and Cl^-, is less complicated than for some which follow, in that these at least are measured in the compartment that contains most of the quantity of each present in the body, namely, the extracellular compartment. Two factors complicate the interpretation of their serum levels: ignorance as to the total extracellular volume, as we have seen; and for sodium, ignorance as to the amount that is present in bone or has entered cells. The amount normally present in bone is nearly 40% of that present in the whole body. This sodium, at least in animals, tends to be released from the bone in metabolic acidosis but much less readily in sodium depletion or in respiratory acidosis. The utility of the Na^+ and Cl^- analyses must be considered again in Chaps. 6 and 10.

LIBRARY PROBLEMS

1. Criticize this statement quoted from a textbook of clinical biochemistry: "The normal [renal] threshold value for NaCl is 560 to 570 mg. per 100 cc. of blood plasma." Criticize also a journal article in which the

value for the average NaCl content of ascitic fluid is given as 642 mg. per 100 ml. What gives the following sentence an archaic flavor? "The urine chlorides were 110 milliequivalents per day."

2. The level of the cerebrospinal fluid chloride was formerly believed to be valuable in the diagnosis of tuberculous meningitis, etc. Examine various editions of a textbook of clinical pathology to see if this view is included. Before deciding what a low cerebrospinal fluid chloride means, what serum analysis would you like to have? Find a treatise on the cerebrospinal fluid in which the diagnostic usefulness of the cerebrospinal fluid chloride analysis is effectively questioned. Is the normal chloride level of the cerebrospinal fluid what you would expect for an ultrafiltrate of plasma?
3. In two cases discussed in the *New England J. Med.*, **255**, 349 (1956), the total 24-hr. excretion of solutes was recorded. How do you suppose this value was determined? How large a fraction of the 24-hr. excretion was accounted for by $Na^+ + K^+ + Cl^-$? Mention other probably important contributors.
4. In case 42221, *New England J. Med.*, **254**, 1035 (1956), the total serum solutes are stated at one point to have been 465 milliosmoles per liter, whereas the Cl^- and Na^+ are said to be 110 and 129 milliequivalents per liter. Are these values internally consistent? In case 41382, *New England J. Med.*, **253**, 526 (1955), the discusser objects to a statement about the patient's "serum water." Reword the statement to meet this objection. What does he mean when he says that the serum Na^+ alone told him as much?
5. The infant is more readily dehydrated by vomiting or diarrhea than the adult. Discuss why this should be the case.
6. For the cases reported in *New England J. Med.*, **253**, 288 (1955), and **256**, 223 (1957), sketch such portions of the serum electrolyte framework as you are able and make predictions about the bicarbonate ion concentration. Make a similar sketch for the values recorded in *New England J. Med.*, **245**, 341 (1951), and draw a conclusion about the presence of an anion for which no analysis is reported.
7. Examine, for example, in Peters and Van Slyke, *Quantitative Clinical Chemistry*, vol. II, *Interpretations* (1931), the history of the idea of *dry storage* of chloride; for example, in pneumonia. Consider whether the idea of *storage* ever tends to assist our thinking about chloride metabolism. Frequently one hears the body *storage* of nutrients compared; for example, for thiamine and ascorbic acid. The term often hides our ignorance of the distributional factor permitting retention. Suggest a substance for which the term *storage* is really objection-free when applied to the body distribution.
8. The low serum chloride in case 43051, *New England J. Med.*, **256**, 223 (1957), was tentatively attributed to a dietary deficiency. What do

you think of this suggestion? Why do you think the hemoglobin and blood NPN levels were high in case 44162, *New England J. Med.*, **286**, 806 (1958)?

9. Investigate and report the use of the sodium or chloride analysis of the sweat for the diagnosis of fibrocystic disease of the pancreas. How can one proceed so as to be sure that the high salinity of a sweat specimen is caused by the disease rather than by partial evaporation of the sweat?
10. Report classical studies by Gamble and associates on the effects of ligation of the alimentary canal at various levels.

4

POTASSIUM DISTRIBUTION

Interpretation of diagnostic potassium determinations introduces a distributional complexity not encountered with Na^+ or Cl^-: We analyze for the concentration in a compartment that contains only a minor part of the K in the body. A biopsy of *muscle* or *liver* would tell us much more about the state of the body potassium economy.

We need to remember, however, that even if available, such a biopsy sample would not be a sample of the pure cellular compartment. Instead it is a combination of the *extracellular* and *cellular* compartments in an uncertain proportion; typically about one-third of the water may be extracellular and two-thirds cellular, but the proportion is not constant from tissue to tissue. Such a specimen is found on analysis to contain a certain amount of Na, somewhat less Cl, and somewhat more K. Historically almost a century was required to realize what these analyses meant; they became rational and useful only when it was realized that they were consistent with the presence of almost all the Cl^- and Na^+ in the extracellular phase and almost all the K^+ in the cellular phase. Even supposing that we could obtain tissue K^+ determinations routinely, some measure of the proportions of cellular and extracellular phase present in the sample would be needed to make the results comprehensible.

The analysis of cells like the erythrocytes, which can be packed by centrifuging so that almost all the extracellular fluid is excluded, gives more straightforward results. Figure 4.1 shows an approximate ion picture for human erythrocytes. But the red blood cells are atypical cells, with very limited metabolic abilities, and their value as a biopsy sample accordingly has been subject to question.

The ion picture of the muscle cell is also approximated in Fig. 4.1. These results have been obtained by assuming that the muscle cell contains a negligible amount of Cl^-. Therefore the Cl^- analysis of the muscle sample is used to calculate how much of the water, Na^+, K^+, and so on, should be attributed to the cells. The cell level of Na^+ calculated in this way is small. Here again the concentrations must be given in milliequivalents *per kilogram of cellular water* (or sometimes per kilogram of fat-free solids) to be meaningful.

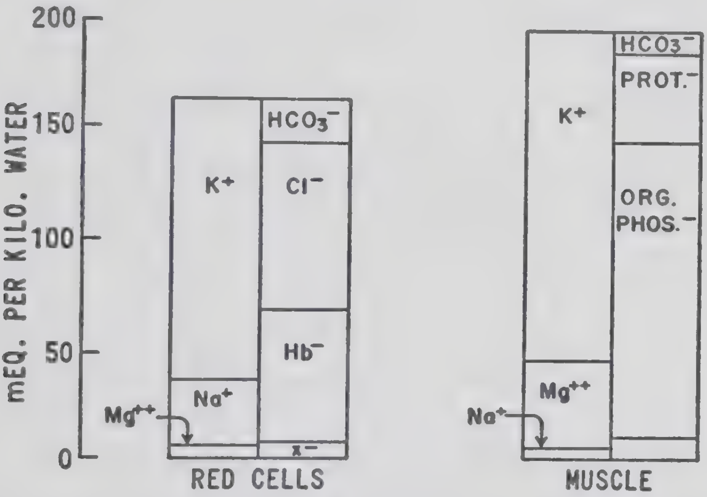

Fig. 4.1 Approximate picture of the electrolyte framework of human erythrocytes and of the cellular phase of muscle. (After Hastings, Harvey Lectures, **36**, 91 [1940–41]).

Potassium and *magnesium* are seen to be the principal cellular *cations*. Among the *anions*, the cellular *bicarbonate* has been measured indirectly. In addition to this anion the cell is very rich in complex anions, such as phosphate derivatives ranging from simple esters and amides to the larger-moleculed phospholipids and nucleic acids, and also the protein anions. Most of the proteins contain considerably more of the acidic amino acids whose side chains contribute a carboxyl group than of those that contribute side-chain amino groups. Therefore, at near neutral pH values, the proteins have an excess of negative charges over positive and are polyvalent anions. We have only crudely approximated here the contribution of each of these two, the carboxylate and phosphate anions, to the total. Sulfate also represents an appreciable fraction of the cell anions. Again we need not doubt that the anions will equal the cations in total concentration.

If we begin from the fact that the principal catalysts and structural elements of the body are large-moleculed *anions*, perhaps we can understand why our body fluids must be salt-containing. If these are to be anions, then cations must also be present; why the cations must be K^+ and Mg^{++} is another question. If we must have an electrolyte-containing solution *within* our cells, the simplest arrangement is to have another of equal osmotic pressure on the outside. Of course the elaboration of an acidic or alkaline digestive or renal secretion is also much simpler if we begin with a salt solution rather than with pure water.

We do not know how much of the magnesium ion of muscle or liver cells is actually free, but presumably the binding of this ion eliminates as many anionic as cationic charges. Even taking this uncertainty into account, the free cation concentration of muscle cells, for example, appears to be larger than that for the extracellular fluid. Does this mean that the osmotic pressure is necessarily higher inside the cells? Which anions contribute least to the total osmotic pressure, per equivalent of charge?

Na^+ and K^+ Discrimination. Active Transport. The question as to how cells discriminate between sodium and potassium ions, so as to *exclude* one almost entirely and to *include* the other selectively, is probably the most embarrassing unsolved biochemical problem of the present. At one time the problem seemed simpler because we supposed that K^+ was simply walled off inside the cells. Now we know from isotope experiments that it is constantly leaking out and the sodium is leaking in, and that the strange distribution is maintained only by constant transfer of the ions against concentration gradients.

Understanding this type of activity is of particular importance to us if we are to approach clinical chemistry from the point of view of *distribution*. Many other possibilities having been eliminated, investigators are coming to regard it likely that there is a *carrier,* a chemical substance that combines with Na+, for example, to form a complex which permeates a barrier. If a gradient is to be created, somehow energy must be imparted to cause the carrier to transfer Na+ in the *outward* direction, against the gradient.

Suppose in Fig. 4.2 we have the carrier C− being generated at the inside limit of the membrane, and perhaps destroyed at the outside limit. If we create a sufficient gradient of C− in this direction, the complex NaC will show net movement outward despite the opposing Na+ gradient.

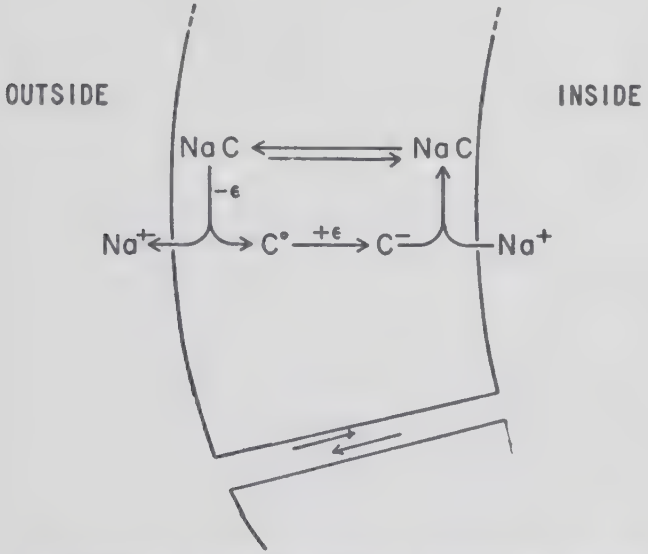

Fig. 4.2 A diagram to represent possible operation of carrier transport. The sodium-carrier complex is caused to dissociate at the outer limit of the cell membrane by modifying C, in this case by removing an electron from it. Reversal of this change can cause another Na+ to be complexed at the inner limit.

One of the ways in which we could form C− would be by adding an electron to some precursor C°, a much weaker sodium binder. If we had an arrangement for again removing the electron at the outside limit of the membrane, then we could maintain the desired gradient of the carrier. As C− is reconverted to C°, the complex NaC will dissociate. This shows as one possibility that the biological electron-transport chain could operate to energize such pumping of solutes. Note that the binding of Na+ to C− must not be merely electrostatic attraction, like that between Na^+ and Cl^-, because no specificity can be provided by such a bond; instead a new molecular species must be formed. Figure 4.3 attempts a hydrostatic model for active transport.

In actuality the use of isotopes has caused us to overestimate greatly the speed of active transport into cells and of leakage out. In fact more energy than is available to the cell for all its functions would be necessary to keep up with the exchange rates often found. This is the same major disappointment attending so many isotopic investigations, the recording of a one-for-one exchange that cannot contribute to net migration. In the case of transport this phenomenon is called exchange diffusion; it is a consequence of the likelihood that a good many of the excursions of the carrier C will be ineffectual and merely return the carried substance to the phase from which it originated, but with an exchange in the meantime for a molecule of the same substance from the other phase. Despite this difficulty with tracer measurements, the necessity for constant transport activity appears unquestionable.

For chloride-excluding tissues like nerve and muscle, pumping-out of Na^+ alone may be adequate to explain the peculiar distribution of Na^+ and K^+ because K^+ would enter to take the place of the extruded Na^+, driven in by the potential difference created by Na^+ removal. In these tissues Na^+ and K^+ movements are probably responsible for the potential changes during activity. If a cell is permeable to chloride, however, as are the erythrocyte and leukocyte, we shall also expect to see Cl^- coming out with the Na^+, rather than K^+ migration alone serving to maintain electroneutrality. If K^+ and Cl^- both migrate freely, their distribution ratios

$$\frac{[K^+]_i}{[K^+]_0} \quad \text{and} \quad \frac{[Cl^-]_0}{[Cl^-]_i}$$

should be equal. Since this is not the case, we believe that at least some cells carry out both, pumping-out of sodium and pumping-in of potassium.

The question that we must consider from here onward is this: To what extent does the cellular K^+ concentration vary with the

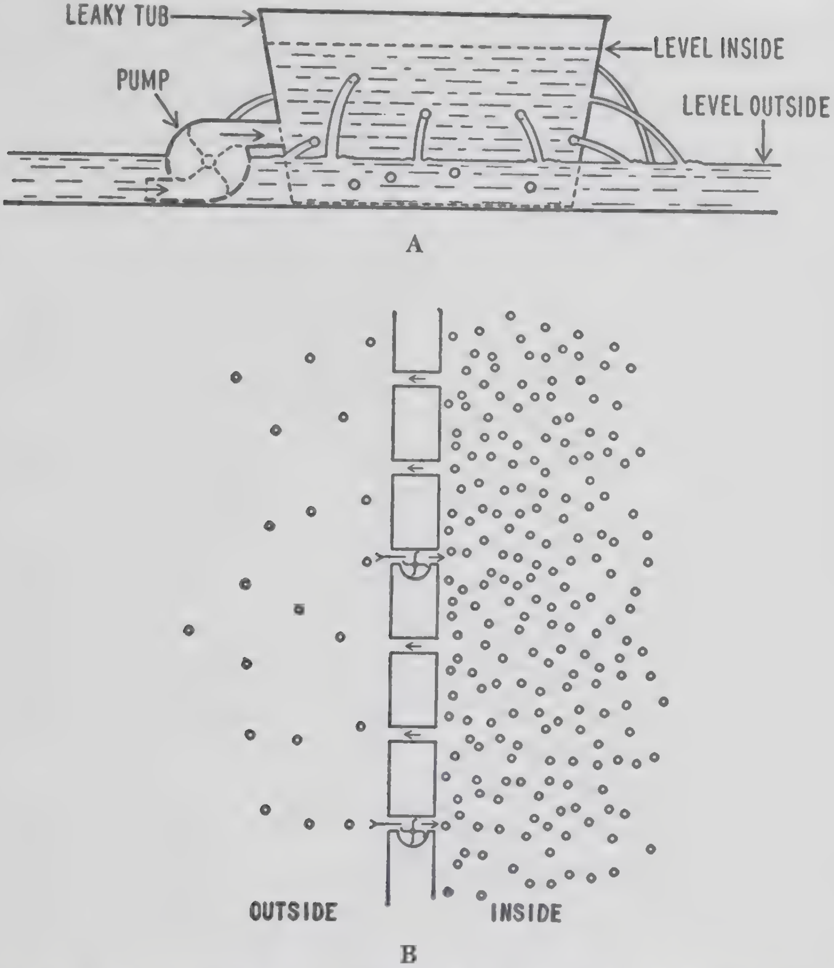

Fig. 4.3 A hydrostatic visualization of the pumping of a constituent into a cell. Part A. The model is supposed to represent the following features: (1) Constant work is necessary to keep the water in the tub at the normal level; (2) the leakiness cannot be unlimited or the pumping will accomplish no significant effect; (3) the lower the level outside and the higher the level inside, the more work is involved in the pumping; (4) exchange between the water outside and the water inside can occur through the pump itself; the higher the level inside, the faster this can occur. The factor of *convection* in modifying fluxes unduly is an unsatisfactory aspect of the diagram.

Part B. To avoid this difficulty, this second model shows instead a pump that is able to transfer the molecules of the solute shown against a concentration gradient. Now one can see more clearly that, through thermal motion, the molecules will leak *in* as well as *out* through the holes. Of course the selective character of the pump for a particular solute molecule cannot be illustrated by this simple model.

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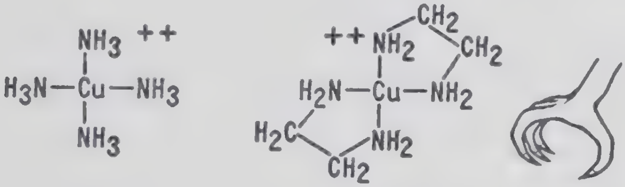
extracellular? Is the situation like the one described for brain alcohol, where we can predict the level at the effective site from the level in the blood? This question is not limited to potassium. There are other solutes, like the amino acids, for which cells show active transport, in this case also concentrating them inwardly. The renal tubular cells and the mucosal cells of the small intestine transfer a great variety of solutes against concentration gradients. Obviously this is a question of broad importance in biochemical studies.

Extracellular K^+. Even though most of the body potassium is in the cells, the minor portion that is outside the cells is very important for their activity and survival. Strangely, the fluid that a cell elaborates as its *interior* environment is *lethal* if it becomes the *exterior* medium. For normal function the K^+ must be far lower on the outside than in the inside, but still a definite amount must be present. The range compatible with normal function is probably narrower than from 3 to 7 milliequivalents per liter in man; usually the serum K^+ normally remains between 4 and 5.5 milliequivalents per liter. Muscle and nerve irritability are strongly modified by changes of the extracellular K^+ level. The heart muscle is most critical in this regard: Below about 3 milliequivalents per liter the heart muscle is likely to be injured; above 7 to 9 milliequivalents cardiac arrest becomes likely. The characteristic changes in the electrocardiogram are important evidences of hypo- or hyperkalemia.

Cellular Potassium. Within the cell, potassium as a cation permits large concentrations of *anionic* catalytic and structural elements to exist and function. Among the great number of cellular enzymes that have been separated, many have been found to be more active in K^+-containing solutions than in Na^+-containing solutions. Now, to the chemist, Na^+ and K^+ are much alike, and both are rather inactive in combining with other structures. We can easily understand from the tendency of Ca^{++} and Mg^{++} (and many other metal ions) to "chelate," i.e., to enter into cyclic combinations like Ca citrate or Ca proteinate (Fig. 4.4), why neither of these serves suitably as the principal cellular cation. Perhaps the slightly greater tendency of Na^+ to chelate is also sufficient to interfere with many cellular enzymatic reactions, or perhaps a slight reactivity of K^+

with certain proteins is essential. One structure is recognized which selectively binds *potassium,* namely, a submitochondrial particulate capable of oxidative phosphorylation (Gamble). Any compound that binds one of these ions selectively should be considered as possibly operating in their differential transport.

Although K^+ is necessarily the main cellular cation, considerable Na^+ substitution is tolerated. In the preceding chapter dehydration was defined in terms of *extracellular* electrolyte alone. For a decade the accompanying *cellular* electrolyte losses were mainly neglected. The extracellular fluid was supposed largely to cushion the cellular compartment from modification, although analytical results have been available since 1933 to dispute this conclusion.



A Complex A Chelate

Fig. 4.4 Complexes and chelates. Chelates are special complexes in which at least one ring-structure has been formed (*chelate* from *chela,* claw).

In that year Atchley, Loeb, et al., reported extensive balance studies on diabetic patients who were permitted to go into acidosis under very complete observation. One patient showed net losses somewhat as follows:

216 milliequivalents of Na^+
362 milliequivalents of potassium

If the negative Na^+ balance represented accurately the loss of extracellular fluid, then a loss of 142 milliequivalents would mean that a liter of this fluid had been lost, and therefore 216 milliequivalents would indicate a loss of only 1.5 l. of extracellular fluid.

Can we go further and say that the negative K^+ balance establishes that $362 \div 150$, or 2.4 l. of cell fluid had also been lost? What would such a loss mean in terms of protoplasmic breakdown? The nitrogen and phosphorus, etc., corresponding to 2.4 l. of cell fluid, or about 10 lb. of tissue mass, can be calculated, and these amounts should also appear in the excreta to confirm this wasting of the patient. That is, the breakdown of tissues is signalized by losses of K, N, and P in the proportions in which they occur in the tissues.

This patient did not, however, lose N and P to correspond to his K loss. This means that the tissues may lose K^+ without breaking down (and presumably therefore also without losing a proportionate amount of complex organic anion). How can a cation be lost without anion loss? The explanation that Na^+ entered the cells in place of K^+ has since been established by careful balance studies.

This means that our preceding calculation *overestimated* the cell-phase loss at 2.4 kilos and *underestimated* the extracellular fluid loss at 1.5 l. In addition to the Na^+ lost from the body, additional Na^+ had also been "lost" *into the cells*. Of course some Na^+ may also have been released from the skeleton. These complexities are not readily taken into account when we rely upon serum Na^+ and K^+ analyses. The technique of the balance study, comparing output with intake, is a valuable supplement but rarely practical in routine diagnosis.

The foregoing experiment involved acidosis as well as dehydration, but similar relationships have been clearly shown without acidosis, notably in the life-raft survival experiments of Gamble and Butler. Volunteers on a six-day fast had their negative sodium balances controlled by giving them NaCl, but this only intensified their potassium losses, which again ran far ahead of the nitrogen losses. From such experiments and many subsequent observations, the body potassium, despite its being "sequestered" in cells, is recognized to be about as subject to depletion in disease as is the extracellular electrolyte.

Control of Urinary K^+ Excretion. The K^+ that passes through the glomerular filter is probably largely reabsorbed in the proximal tubule. As discussed in the preceding chapter, the principal K^+ excretion occurs in the distal tubule; here a complex exchange pump has been proposed whereby K^+ and H^+ compete for Na^+ exchange from the tubular lumen. For example, when the plasma hydrogen-ion concentration is lowered, K^+ excretion is increased. Conversely, when K^+ excretion is increased by feeding potassium salts, the urine becomes more alkaline. One may suppose that this arrangement would handicap the kidney in disposing of independently variable loads of H^+ and K^+. The rather poor conservation of K^+ when the intake is low may point to this handicap.

As we shall discuss presently, the presumed cellular K^+ pumps in muscle and liver show similar indications of competition between H^+ and K^+ for transfer, so that K^+ tends to be displaced in acidosis.

Because K^+ excretion occurs into the distal tubule, it is not remarkable that the plasma (i.e., glomerular filtrate) K^+ level is not decisive in determining the excretion rate. Instead, the renal pump appears to be more responsive to the total body K^+ supply (perhaps as represented in the tubular cell).

How Potassium Deficiency May Develop. Deficient potassium *intake per se* is rare (except experimentally) as a primary cause of potassium deficiency, although it may be a contributing factor. When there is a deficient intake of K^+, however, the kidney does not stop excreting K^+ nearly so completely as it does for Na^+ when the Na^+ intake is restricted. Nevertheless, for K^+, we again have mainly *increased losses* to consider. These we may divide into three categories:

1. *Increased* K *Excretion the Primary Cause.* This causation can be illustrated by the severe K deficiencies that develop in diarrhea, especially infantile diarrhea. Darrow's studies of such patients played a large part in bringing appropriate attention to cellular electrolyte depletion. He found that children, during recovery from severe diarrheas, built up substantial positive balances of K^+; this could only mean that earlier they had been severely depleted.

Why should potassium be abundant in diarrheal stools? Ordinarily the mucosa of the lower intestine reabsorbs electrolytes and water so completely that we are not concerned with the precise behavior of this mucosa with regard to each ion. By instillation of saline solutions, however, it has been established that this mucosa enters into quite a different steady state with such solutions than does the duodenal mucosa. The instilled fluid tends to be enriched in potassium and depleted in sodium during its absorption until the K^+ levels are substantially higher than those of the extracellular fluid. When disease interfers with the resorption of the fluid, this K^+-enriching activity can lead to dangerous potassium losses. Vomiting is another important cause of potassium depletion, and intestinal fistulae may also contribute to potassium loss.

Excessive potassium losses in the *urine* may be caused by action of abnormal amounts of "salt-retaining" adrenal steroids on the kidney. Deoxycorticosterone and other therapeutic steroids like cortisone carry this hazard. Or if the adrenal cortex secretes unusual amounts of such hormones, as in *Cushing's syndrome,* potassium deficiency will be produced unless the potassium intake is sufficiently increased to compensate for the wasting. An aldosterone-secreting adrenal tumor (primary aldosteronism) causes serious potassium wasting (Conn). At the present time a substantial volume of inorganic ion analyses are requisitioned to protect the patient from excessive electrolyte changes arising from the therapeutic use of steroids.

Finally, the kidney tubule itself may possibly be at fault in conserving potassium. A group of associated defects in tubular conservation includes a defective ability in excreting hydrogen ions, which places drains on various other cations, for reasons that will be made clearer in Chap. 8. The calcium economy is likely to be more severely compromised, but K-wasting is often considered a significant aspect.

Urinary potassium analyses represent an important mode of determining whether depletion is occurring by this route. If the losses have another pathway, a low urinary K^+ excretion rate should be found.

2. *Increased* K *Excretion Arising from a Shift of Cellular* K^+ *to the Extracellular Fluid.* With over 3000 milliequivalents of our potassium in the cells and only about 70 milliequivalents in the extracellular fluid (Fig. 4.5), one can easily understand that a minor upset in the normal K^+-pumping by our cells could flood our extracellular fluid with a lethal K^+ level, were it not for the constant activity of the kidney in controlling the extent of tubular excretion of K^+. A corollary is that the amount of potassium retained in the body is determined quite as completely by the behavior of the barrier (or pump), which determines how much of the K is retained

in the cells, as by the renal barrier that determines how much of the extracellular K^+ is retained in the extracellular fluid. Any slowing of the cellular pumps in general will cause K^+ wastage.

Although not so well studied, cation-pumping in the cells of muscle and liver is very probably also under hormonal control, as in the renal tubule, and many of the cation shifts of disease will probably be found to have an endocrinologic origin. Cells may also lose potassium by mechanisms that are probably nonendocrinologic. For example, if we starve when our glycogen stores are depleted, the equilibrium quantities of metabolites pursuing catabolic and anabolic courses will be changed; presumably the concentration of sugar phosphates and other organic anions will be decreased, especially in the liver. Therefore, even if the cation pumps are working as strongly as ever, cations must be lost from the cell to the extracellular fluid, and thence from the body. The disappearance of the liver glycogen in particular has come to be associated with potassium losses. Also, in dehydration and in acidosis, we have seen that K^+ tends to be lost from the cells with Na^+ replacement for incompletely known reasons. If the lost extracellular NaCl is vigorously replaced, this tendency understandably is further intensified.

3. *A Shift of the Potassium into the Cells Causing Hypokalemia.* This cannot properly be labeled a K^+ loss, yet it is an important factor in the appearance of K^+ deficiency. In the situations described in the preceding paragraphs, the cellular potassium deficiency *per se* may appear to be comparatively innocuous and latent. If, however, a return of potassium to its normal distribution is set in motion, the *extracellular* K^+ level is quickly reduced to dangerous levels. The character of the equilibrium set up does not, of course, permit a complete removal of K^+ from the extracellular fluid, but K^+ may be brought down to lethal levels. Correction of the earlier defect (for example, by rehydration, by glycogen resynthesis and accelerated glucose metabolism, as in treated diabetic coma) will uncover and make dangerous the latent K^+ deficiency. (Glycogen is completely unassociated with K^+ in the granules in which it is deposited in the liver; nevertheless the close association between

glycogen resynthesis and potassium re-entry sometimes has led to the fallacious idea that these two substances combine.)

Hypokalemia without an over-all potassium deficiency can be produced by testosterone therapy, whereby an intensified transfer of potassium into cells is produced. Clearly, however, the shift of potassium into the cells is a hazard that usually arises secondarily to a pre-existing K^+ depletion. The most frequent sequence of events undoubtedly is mechanism (2) followed by mechanism (3).

Relationship of Potassium Deficiency to Alkalosis. Darrow observed that children recovering from diarrhea frequently showed a persistent alkalosis, which disappeared, however, when potassium was given. Alkalosis has also been recognized in other potassium deficiencies; for example, in Cushing's syndrome. In an attempt to explain this curious association between K^+ deficiency and alkalosis, Darrow and his associates caused small animals to become alkalotic by withholding K from their diets. These animals had their alkalosis corrected by KCl, but instead of showing urinary changes consistent with the correction of *alkalosis,* they showed changes of *the opposite character,* as follows:

Increased urine titratable acidity
Increased ammonia excretion
Decreased urinary bicarbonate

These findings left little doubt that the convalescing animals were combating an *acidosis* rather than an *alkalosis!* Furthermore the restoration of K^+ to rats previously made alkalotic by K^+ restriction *lowers their plasma pH in the absence of the kidneys,* establishing the endogenous origin of the hydrogen ions returned to the plasma (Orloff).

This paradox has been explained tentatively as follows: The observations leading to the diagnosis of alkalosis were made upon the *extracellular* compartment. In potassium deficiency the usual association between the pH of the extracellular and cellular compartments is believed to be lost; instead, as Na^+ replaced cellular K^+, approximately *one* H^+ accompanied *two* sodium ions in replacing *three* potassium ions, thereby *acidifying* the cellular compartment and *alkalinizing* the extracellular. In potassium-depleted rats, indirect methods have indicated a drop in the pH calculated for the muscle fiber interior from 7.0 to 6.4 (Gardner). If a whole-body acidosis is produced, then the kidney must be retaining more H^+ than normally, which is the appropriate response to an alkalosis. In this case, however, the response *fails* to correct the alkalosis because the potassium deficiency restrains the hydrogen-ion retention, below that normally expected for the given degree of alkalosis, through the reciprocal relationship between H^+ and K^+ excretions. As a result an extracellular alkalosis remains as long as K^+ deficiency continues. Prolonged K^+ deficiency has been shown to cause characteristic renal lesions as well as cardiac injury. The characteristic "kaliopenic" nephropathy prevents the usual response to the anti-diuretic hormone, so that the urine cannot be concentrated normally.

The foregoing conclusion is a disturbing one that has not yet been fully evaluated. Later we shall see that excess H^+ in the body is ordinarily buffered partially in the cells, partially outside. We are led to wonder: Are there other situations in which the assumed association between hydrogen-ion concentration of the cells and of the extracellular fluid does not hold? Also we should like to know whether it is net movement of Na^+ into the cells or the loss of K^+ from them that impels H^+ into the cells or whether a single chemical substance actually transports all three. The effect conceivably could arise from the opposition of the Na pump to Na^+ replacement of the escaping K^+, so that H^+ exchanges instead for part of the K^+.

Periodic paralysis is a familial disease known to be characterized by episodes of hypokalemia. Recent studies show large periodic increases in aldosterone output, accompanied primarily, however, by intense *sodium* retention, with abnormal amounts of sodium entering muscle (Conn). Potassium sequestration in cells then may subsequently lower the plasma K, but this event appears not to be essential to the attack. The muscles come, nevertheless, to contain an abnormal total concentration of the two alkali-metal cations; a characteristic lesion, hydropic vacuolization, has been associated with this strange, poorly understood situation. The stimulus to the intermittent aldosteronism in this inborne disease is unknown, but it appears not to lie in the adrenal glands.

Detection of Potassium Deficiency. From the foregoing sections it should be clear that the extracellular K level does not necessarily mirror the cellular level, since the extracellular pH and the renal function are variable factors affecting the relationship between these two phases. If the serum K^+ is below 3 milliequivalents per liter, there is almost certainly an over-all potassium deficiency because only exceptionally do the K pumps work harder than usual in disease. But if the serum K is normal, only guarded conclusions can be drawn concerning the total body economy. Hence laboratory guidance is frequently unavailable in decisions regarding K replacement.

Balance studies have led to most of our information concerning the likelihood of potassium deficiency in various situations. These appear in general to indicate that K deficiency is almost of parallel likelihood to Na deficiency, but this is usually not interpreted to mean that intravenous K replacement should be as routine as replacement of extracellular electrolyte. The difficulty is not merely the absence of a reliable measure of the K deficiency but also the awkwardness and danger inherent in the necessary route of K replacement: All the K^+ *must pass through the extracellular compartment,* with its mere 70 milliequivalents of total K^+ (Fig. 4.5), and yet add significantly to the thousands of milliequivalents in the cells. For safety this transfer must occur without increasing the plasma K^+ by more than 2 or 3 milliequivalents per liter. Obviously a "balanced" solution containing 5 milliequivalents of K per liter is almost worthless for K^+ replacement. Solutions containing as high as 40 or even 60 milliequivalents per liter are successfully infused, demonstrating the speed with which the various cells may remove the extra potassium added to the extracellular compartment. One-half this concentration is considered safer, and such infusions are made under close observation of the adequacy of urine formation.

Incidentally, K^+ is not the only cellular electrolyte that may be neglected in an enthusiasm for "physiological" saline. During a glucose infusion or a glucose tolerance test, the serum *inorganic* *phosphate* is markedly reduced through cellular uptake. When carbohydrate catabolism returns to a normal rate in treated diabetic coma, similar large removals of extracellular phosphate occur. Magnesium deficiencies have also been observed in diabetic acidosis and in patients treated with parenteral fluids.

Our food is rich in potassium and certainly represents an ideal route of replacement, providing that this route becomes available quickly enough. The urgency of potassium replacement increases as the period of time lengthens during which a patient must be maintained on intravenous nutrition.

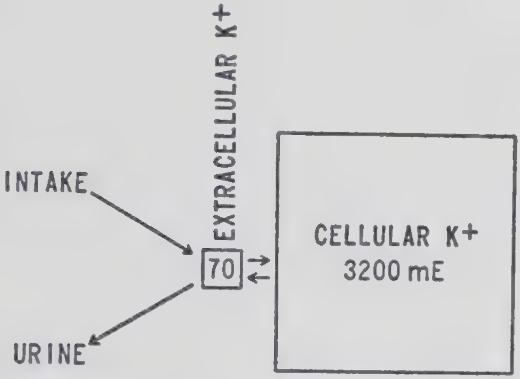


Fig. 4.5 Illustrating roughly the comparative sizes of the extracellular fluid potassium and the cellular potassium pools. Maintenance of the potassium supply of the body depends not only upon the equilibrium between urinary and plasma K+ but also upon the potassium-concentrating process of all the cells of the body. Certain factors that cause loss of K+ from the cells (for example, lowered extracellular pH) also interfere with K+ excretion, thereby restraining somewhat the loss of K+ from the body when K+ is being lost from the cells. Repletion of body K+ must take place via the small extracellular pool without greatly increasing it.

Hyperkalemia. Ordinarily the kidneys excrete whatever excess of K+ we ingest, so that the hazard of hyperkalemia appears to be restricted to the injection of potassium-containing solutions and to kidney disease. Especially in acute renal failure (acute tubular necrosis, lower-nephron nephrosis), which may result from intravascular hemolysis or crush injuries, the serum K level rises during the anuric or oliguric phase, and survival depends upon avoiding the cardiac arrest that may be produced by hyperkalemia. No special source of extra K^+ may be necessary for this hyperkalemia, again considering the great size of the cellular reservoir compared with that of the extracellular (Fig. 4.5). Because the long-term prognosis is frequently favorable, removal of extracellular K^+ by the "artificial kidney" or by ion-exchange resins may be lifesaving. In the first of these, arterial blood is dialyzed against an initially potassium-free, artificial extracellular fluid and then reintroduced into a vein. In the second, a polymeric carboxylic acid in granular form is introduced as a retention enema. Potassium ions exchange for the hydrogen ions of the resin. The resin may instead be used as an NH_4^+ salt.

Other measures for restricting the rise of the serum K^+ include the administration of glucose, insulin, and alkali.

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1. Investigate the use that has been made of radioactive potassium to discover the K^+ content of the body. Is all the body K^+ exchangeable with administered K^{41}? Evaluate this as a practical diagnostic method.
2. Show carefully that a sodium pump alone without a potassium pump is not adequate to explain the behavior of the ions of the erythrocytes.
3. Cite the instance of the substance most powerfully concentrated by active biological transport. Is the mode of its transport clearly understood? Is the mode of hydrogen ion "pumping" through the renal tubular cell understood? The source of the H^+ may have been identified, but is the form in which the H^+ is carried through the osmotic barrier known?
4. One of the methods of detecting how an organic molecule has been grasped during its biological transport is to look for a subtle change in a part of the molecule, like the replacement of an atom by its isotope, which would not have occurred had the substance not been transported. This may show whether the atom concerned is involved in linkage of the transported molecule to the carrier. Another method is to make a small structural change that prevents grasping at that point and then to see if transport is still possible. These methods are not available for solutes like Na^+ and K^+. Attempt to find illustrations of the use of each of these methods.
5. Is potassium the chief electrolyte of the erythrocytes of all species? What happens to the cations of human whole blood that is chilled some hours and then brought back to body temperature? Is oxygen always necessary for active transport?

6. Textbooks have frequently spoken of the neutralization of NaOH added to an isoelectric protein; for example, hemoglobin, as "base-binding." Which of the following is the better description of what actually occurs?

   a. The sodium ion becomes bound to the protein molecule.
   b. The added hydroxyl ion removes a hydrogen ion from a dissociating group of the protein; for example, from an amino group. The result is that the protein loses a positive charge and hence becomes an anion.

   Have any studies been made to prove that hemoglobin, for example, does not actually bind Na^+ or K^+?
7. Why is the negative P balance not so convenient as the negative N balance for estimating tissue breakdown? How may one correct for the source of uncertainty encountered here?
8. Tabulate the compositions of the replacement solutions recommended by Hartman, by Darrow, and by Butler. Discuss the differences among them.
9. Find and discuss a paper showing renal injury consequent to potassium deficiency.
10. Find and report articles investigating the limitation of protein synthesis or repletion in potassium deficiency.
11. Find and report a paper on the potassium-binding behavior of submitochondrial particulates derived from mitochondria.
12. Report on the biological substitution of Na^+ by lithium or of K^+ by rubidium.
13. Report on factors involved in the composition of the saline used in the "artificial kidney."
14. Find and report a paper evaluating the red blood cell as a biopsy sample for investigating the cellular potassium supply of the body.
15. Demonstrate the method of Lowry and Hastings for calculating the cellular water and extracellular water of a tissue sample upon the basis of electrolyte and total water determinations.

5

CALCIUM AND PHOSPHATE DISTRIBUTION

The distribution of calcium and phosphate is dominated by the interaction that they show when they are brought together. Over 99% of the calcium and 83% of the phosphorus of the human organism are contained in the skeleton and the teeth; the behavior of the minor portions present in the body fluids can be understood only with reference to this huge skeletal reservoir.

This is not to say that the small percentages present in soft tissues are not important. The calcium in our extracellular fluids is critical for the activity of nerve and muscle, especially for the amplitude of the contraction of the heart muscle. Furthermore, Ca^{++} is necessary for the coagulation of the blood. The phosphorus not in the skeleton plays even more varied roles: Its ability to be excreted either as $H_2PO_4^-$ or $HPO_4^=$ gives broad latitude in the quantity of hydrogen ion excreted; it serves in many vital cellular structures and in the degradation and synthesis of numerous carbon compounds; finally, it serves to retain the energy of the oxidation and breakdown of many substances in a quickly available form, the so-called *high-energy phosphate bond.*

Bone Formation. This process occurs in a matrix, which is a gel formed by a network of collagen fibers with the cement substance *chondroitin sulfate* dispersed among the fibers. The inorganic crystals are laid down along the collagen fibers. Around the spicules of newly formed bone *osteoblasts* are seen. We say these cells are forming bone, but actually this is not yet a meaningful statement. Similarly we see cells of the osteoclast morphology where bone dissolution is prominent, but their contribution is not clear.

Bone is a microcrystalline salt, showing upon x-ray bombardment a very strong diffraction pattern shared with a considerable group of minerals (hydroxyapatites) and with precipitated tricalcium phosphate. Nevertheless bone does not have a constant composition; here again it resembles the synthetic precipitates. These crystals apparently admit and adsorb various ionic groups without change in the geometry of the crystal lattice; hence bone contains, besides Ca and P, inconstant proportions of carbonate, fluoride, citrate, sodium, potassium, and magnesium. In addition the tendency of a great variety of heavy-metal elements to be deposited in the bone presents toxicologic and radiologic safety problems.

Bone is precipitated from solution; the mineral is brought to the site in solution in the extracellular fluid. We can be sure that no precipitation of bone crystals can occur unless the concentrations of the participating ions in the immediate environment exceed certain values as governed by the solubility-product principle; the crystals cannot dissolve unless the participating ions fall below such values. The general behavior of bone in forming and dissolving confirms our confidence that these events will be governed by the concentration of calcium and phosphate ions, but the precise nature of the relationships are far from simple. First we should ask the question: Are our extracellular fluids *just saturated* or *undersaturated* or *supersaturated* with respect to bone?

Ion Concentrations. The serum Ca lies near 10 mg. per 100 ml., or 2.5 millimoles per liter. Oddly, Ringer found this an excessively high calcium concentration for the optimal beat of the isolated frog heart. Instead he chose empirically a 1 millimolar level as ideal. Nevertheless serum was an entirely satisfactory environment for the heart. Careful study has shown that the frog heart is responsive to the ionized part of the calcium only and that it permits a reliable measure of the amount which is free. About one-half of the serum calcium is bound (chelated) to the various serum proteins, but it is in a rapid equilibrium with the other free half. Various proteins enter into different equilibria with Ca, which can be described by the simple equations:

$$\text{Proteinate}^{=} + \text{Ca}^{++} \rightarrow \text{Ca proteinate}$$

$$\frac{(\text{Ca}^{++})(\text{prot.}^{=})}{(\text{Ca proteinate})} = \text{K}$$

In general, as the serum protein level rises, the bound calcium rises in direct proportion, provided that the pattern of proteins present does not change too radically. Various homeostatic mechanisms maintain the ionic calcium at a steady level of slightly over 1 millimolar or 10^{-3} M. The concentration of bound Ca meanwhile varies directly with the protein concentration.

Now the ionic calcium is the functional calcium as far as the heart beat or bone formation is concerned; this is the part of the calcium that has diagnostic significance and which we shall prefer to measure. Unfortunately there is no simple method for doing this. When we add ammonium oxalate to serum, we precipitate not merely the free calcium ion but we also cause the calcium proteinate to dissociate until essentially all the calcium is precipitated. That is, the complex with oxalate is much more stable than that with the serum proteins, so that all the calcium passes to the oxalate, or with citrate addition, to the soluble calcium citrate complex.

Accordingly, in practice, serum calcium levels cannot be interpreted precisely unless we know the protein concentration of the serum also. This principle is frequently ignored, but the result can only be loss of precision of judgment. Suppose a patient has only one-half the normal serum protein level, including normal proportions of the various proteins. For that patient there should be only one-half as much bound calcium as usual; hence *for this patient* a serum calcium level of 7.5 mg. % is *normal*. In such a patient a serum calcium of 10 mg. % could represent a clear indication of parathyroid disease! Here is another of the necessary feedback corrections of the interpretation of one analytical result arising from a different analysis, as mentioned in Chap. 1.

We must emphasize that the complication introduced by protein binding is more analytical than biological (i.e., it is a complication in arriving at the information we really need, the *ionic* calcium). If the plasma albumin is increased by infusion, we shall momentarily tie up some ionic calcium, but this will be rapidly replaced from the reservoirs under the homeostatic influences that keep the ionic calcium concentration steady, so that calcium metabolism will proceed normally. In analogy suppose a bay were suddenly formed on the periphery of a rapidly flowing stream. Momentarily the flow would be diverted, but in a few hours the bay, now filled, would no longer be a factor in the flow. We shall encounter similar situations further on; for example, with reference to the plasma hormonal iodine.

Turning to the inorganic phosphate, the serum of the adult averages about 3 mg. per 100 ml. and the child may have twice as high a level. The adult value represents about 1 millimole per liter. At pH 7.4, 0.6 pH units above the pK,

$$\log \frac{HPO_4^{=}}{H_2PO_4^{-}} = 0.6$$

and therefore

$$\frac{HPO_4^{=}}{H_2PO_4^{-}} = 4$$

Hence about 80% of the plasma phosphate is the secondary ion, $HPO_4^{=}$, and 20% is the primary ion, $H_2PO_4^{-}$.

Now bone does not contain *either* of these ions, but instead the radical $PO_4^{\equiv}$ appears in the precipitate. The equilibrium under description is the same, however, whether we write

$$\left\{\begin{array}{lll} 2HPO_4^{=} & \rightarrow & 2PO_4^{\equiv} + 2H^{+} \\ 3Ca^{++} + 2PO_4^{\equiv} & \rightarrow & Ca_3(PO_4)_2 \end{array}\right\}$$

or

$$3Ca^{++} + 2HPO_4^{=} \rightarrow Ca_3(PO_4)_2 + 2H^{+}$$

Likewise, the intermediate formation of $CaHPO_4$, for example, does not modify the over-all equilibrium if this passes on to tertiary calcium phosphate in the presence of the extracellular fluid. The tertiary phosphate-ion concentration of serum can be calculated from the value of pK_3 of H_3PO_4; the concentration found for this ion is about $10^{-7}M$ at pH 7.4.

Solubility Product Principle. A solution containing silver ions and chloride ions can precipitate AgCl only if the product of the two ions exceeds 10^{-10}. If

$$[Ag^+] \times [Cl^-] = 10^{-8}$$

the solution is *supersaturated* with respect to AgCl, and precipitation tends to occur until the ion product is lowered to 10^{-10}. Supersaturation is not a rare occurrence in biological solutions; the urine and bile are regularly supersaturated in certain constituents.

Assuming $Ca_3(PO_4)_2$ to be of primary importance in bone formation, we can calculate from the preceding Ca^{++} and $PO_4^{\equiv}$ concentrations the corresponding ion product as

$$(Ca^{++})^3 \times (PO_4^{\equiv})^2 = (10^{-3})^3 \times (10^{-7})^2 = 10^{-23}$$

This is an approximation for the ion product for our serum. Now we need to see how this compares with the ion product corresponding to saturation. Efforts to measure the ion product that exists for a solution saturated with $Ca_3(PO_4)_2$ has given much smaller values than this; for example, $10^{-26.4}$. This result suggests that the serum is *supersaturated* with respect to tricalcium phosphate, i.e., that serum contains about 2½ times as much of each ion as would be expected at saturation. A complication in the preceding experiment is the tendency of the precipitate phase to change its composition, when in contact with a saline solution, by exchanging various ions of the lattice; the result is a considerable uncertainty about the true value of the solubility product at equilibrium. A value of $K_{sp} = 10^{-23.1}$ has been obtained for zero solid phase by extrapolation, but validity of this procedure is uncertain. Comparison of the product $(Ca^{++}) \times (HPO_4^{=})$ with the solubility product found for $CaHPO_4$ likewise suggests that our plasma is supersaturated.

Figure 5.1 shows how the pH modifies the level of phosphate required to cause calcification of rachitic cartilage by a synthetic solution containing 0.5 millimolar Ca^{++}. As $H_2PO_4^-$ is converted to $HPO_4^=$ and the latter to $PO_4^{\equiv}$ by a rising pH, a smaller and smaller amount of total inorganic phosphate is required to supply the limiting concentration of the critical ion, whichever it is, $HPO_4^=$ or $PO_4^{\equiv}$.

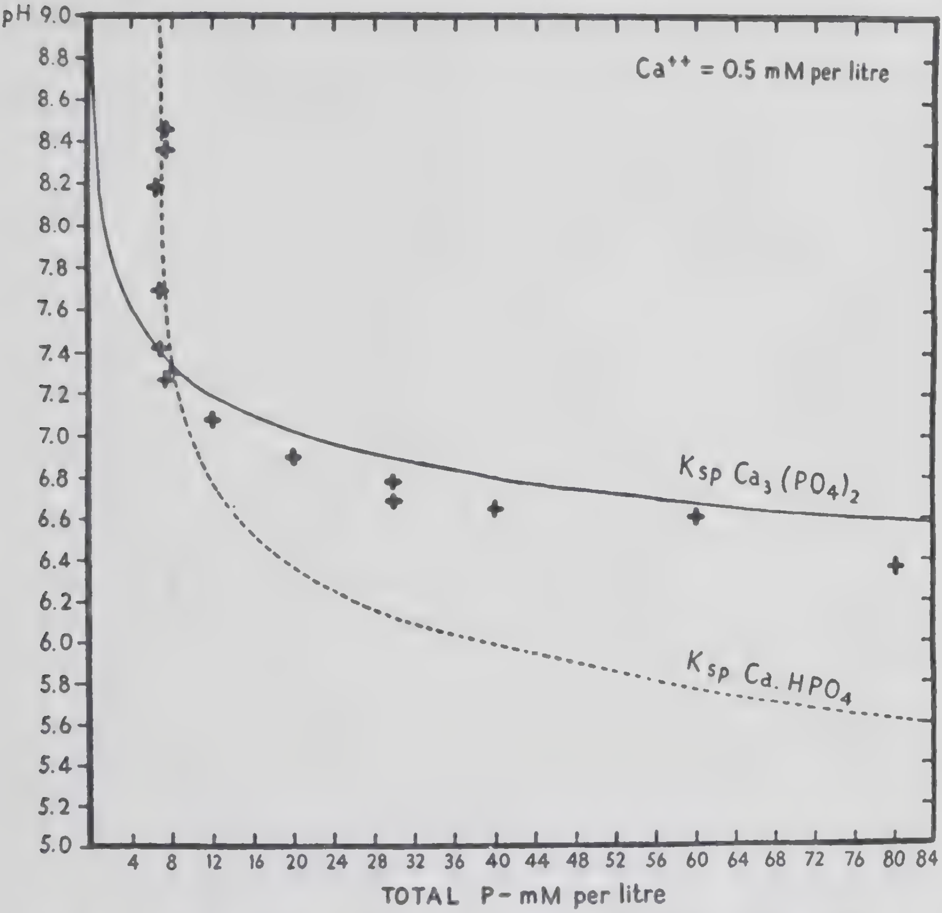

Fig. 5.1 The "limits of biological solubility" for bone. The crosses show the minimal inorganic phosphate concentration required to produce calcification of rachitic cartilage exposed to a 0.5 millimolar calcium-ion concentration. The upper curve shows the prediction of the amount of P required if K_{sp} for $Ca_3(PO_4)_2$ is $10^{-23.1}$. The lower curve shows the relationship expected if $CaHPO_4$ formation were the only significant event concerned in determining solubility. (From F. C. McLean, "Metabolic Aspects of Convalescence." *Transactions of the 14th Macy Conference* [1946], p. 33.)

The solid line in Fig. 5.1 shows that the amount of total phosphorus required should decrease *very rapidly* with pH if $PO_4^{\equiv}$ is the ion concerned. (The actual *position* of this line, as indicated in Fig. 5.1, is open to question.) The dotted line shows that a *slower change* of the total phosphorus requirement with pH would be expected if $HPO_4^{=}$ is the critical ion. The slope actually portrayed by the points shows a closer correspondence to the $Ca_3(PO_4)_2$ requirement except above pH 7.6, where calcification requires an unaccountable concentration of phosphorus, unless we assume that $CaHPO_4$ shifts into a position of prominence in this range. Such a shift does not appear probable because $CaHPO_4$ is stable only at rather low pH values, *not at high ones.* Even if $CaHPO_4$ were a transient intermediate, the subsequent exchange of its H^+ for environmental calcium ion would be expected to cause a sensitivity to pH similar to the solid line of Fig. 5.1.

This situation is not so ambiguous as it may seem. We can expect the experimental points to correspond precisely to the solubility product of tricalcium phosphate, for example, only if the ion concentrations in the suspending fluid or plasma are identical with the ion concentrations at the actual site of crystallization in the cartilage. The following possibilities should be considered:

1. *Perhaps a pH Gradient Is Maintained between the Calcification Site and the Main Body of the Extracellular Fluid.* A pH of 6.8, for example, at the actual site could serve to explain how bone can be dissolved at plasma levels of Ca and P that appear to correspond to supersaturation. Glycolysis could produce a pH gradient (Norden). In order for the solubility product of $CaHPO_4$ to control calcification, apparently we would have to assume that subsequent exchange of the hydrogen ion for calcium occurs without reference to the hydrogen-ion concentration of the extracellular fluid; hence that *secretion of* H^+ occurs.

2. *Perhaps a Gradient of Inorganic Phosphate Is Maintained by Cellular Activity.* Osteoblasts, like so many cells, phosphorylyze glycogen to yield glucose phosphates that can again release inorganic phosphate by phosphatase action. These cells are very rich in an alkaline phosphatase; furthermore, adding a phosphorylase inhibitor interrupts calcification of cartilage slices. This reaction sequence begins and ends with inorganic phosphate and therefore does not have an obvious function in concentrating phosphate in either direction. For this to be accomplished, phosphate must be taken from one compartment and released into another. The osteoblasts do not appear to form a barrier adequate to maintain an intercompartmental gradient. A rhythmic uptake and release of phosphate would be of dubious effect, since the *mean* phosphate concentration

should determine whether net calcification or decalcification occurs.

3. *Perhaps a Net Movement of Citrate (or Some Other Chelator) Occurs from the Calcification Site.* Carbon compounds could move *to* the osteoblasts in the form of glucose or pyruvate and *from* the osteoblasts in the form of citrate, the latter carrying with it bound calcium ion not figuring in the solubility product. The extra citrate could then be destroyed elsewhere; for example, in the kidney. Neuman has shown that blood leaving the spongiosa is richer in citrate than that arriving, and that this difference is further increased by introducing parathyroid hormone into the arterial supply. Therefore the proposal is made that the hormone functions by increasing citrate release.

Two other proposed local effects, perhaps closely related to each other, are not "concentrative" in character: a chelation of calcium by the chondroitin sulfate ground substance, and the provision of unidentified *nucleation centers* for the beginning of crystal formation.

The inevitable dependence of calcification (or at least of decalcification) upon the concentration of the ions concerned, obscured somewhat by local effects, is sufficiently clear to direct the interpretation of diagnostic serum analyses:

a. Factors that lower either the level of Ca^{++} or $PO_4^{\equiv}$ (or $HPO_4^{=}$?) tend to cause bond dissolution. These include acidosis for a reason that should be obvious.
b. Elevating low serum Ca or P *tends* to correct faulty bone formation.
c. Elevating the serum phosphate level *tends* to depress the serum calcium; elevating the serum calcium *tends* to depress the serum phosphate. Precise constancy of the ion product will not be expected if we understand that a tendency toward supersaturation is normal.

Action of the Parathyroid Hormone. Parathyroid extract appears to have two separate actions, although it is well to doubt that one hormone can produce two *unrelated* effects. In fact some evidences suggest that there are two hormones. Foremost, the administration of the extract causes bone to dissolve, thus raising the serum calcium.

Secondly, it inhibits renal tubular phosphate resorption, causing phosphate diuresis, and probably also decreases renal Ca clearance. Much discussion has been given to the possibility that the first effect is entirely caused by the second, but this appears now to be largely precluded because the hormone can raise the serum Ca in nephrectomized animals and produce the histological concomitants of bone dissolution. Furthermore, transplanted parathyroid tissue causes resorption of nearby bone, but other tissues do not. Finally, addition of the hormone causes cartilage slices to decalcify when Ca and P are at levels that would otherwise produce calcification.

Neither the hypercalcemia nor the hypophosphatemia of primary hyperparathyroidism is sufficiently marked to permit uniform discovery of the disease by their determination. An increased phosphate clearance is probably a more reliable diagnostic finding (Kyle).

The output of parathyroid hormone appears to be controlled by the free calcium-ion level of the plasma; any factor lowering this level tends to increase the glandular activity. Prolonged hypocalcemia produces the parathyroid hyperplasia recognized as *secondary hyperparathyroidism.*

Thyroid disease is also marked by negative calcium balances, and in some cases the osteomalacia may be radiologically indistinguishable from that of hyperparathyroidism.

Vitamin D and Dihydrotachysterol. Animals deprived of vitamin D show increased loss of calcium into the feces and defective bone deposition. The action of the vitamin is believed to be predominantly an enhancement of Ca absorption from the intestine, which is especially noticeable when calcium absorption is handicapped by a high phosphate intake. (Why should this disturb Ca absorption?) Evidence for action of vitamin D at the calcification site is also strong; establishment of a unifying explanation (for example, a general action upon calcium transport, perhaps a specific action upon citrate production or oxidation) may be anticipated.

With very large dosages of vitamin D, bone destruction occurs and the serum citrate level rises. At the same time metastatic calcification develops in the soft tissues (calcinosis), especially in the kidneys. Interestingly, the kidney is notably a citrate-destroying tissue. Uremia has been a frequent consequence of calcinosis. This effect of excess vitamin D also argues for a metabolic action not limited to the intestinal wall.

A product of the overirradiation of ergosterol, closely related to vitamin D, is dihydrotachysterol (AT-10), which has a very powerful but brief lytic action on the bone and a pronounced phosphaturic action. It has been useful in alleviating hypocalcemic attacks following parathyroid surgery and is generally used for the treatment of chronic hypoparathyroidism.

Estimation of Calcium Requirement. The adult American who takes in a gram of calcium per day excretes the same amount, about one-third by the urine, two-thirds in the stool. If his intake is cut to 0.5 g. per day, his excretion may continue at perhaps 0.7 g. per day; therefore he is in negative calcium balance. If the intake is again increased to 0.8 g. per day, the output rises to perhaps 0.9 g. The intake must be increased to about 1 g. a day to produce calcium balance. From such observations the recommended daily allowance has been set at 1 g. per day, an amount not readily reached without the use of milk and which is larger than most of the world's inhabitants can provide for themselves.

The premise that has been assumed here is that the organism needs to be kept at his status quo. This assumption appears to be undermined by observations made by Hegsted, et al., on Peruvian prisoners, who had long been on a much lower calcium intake. When their calcium intake was set at 0.4 g. per day, the excretion was 0.4 g. Lowering the intake to 0.3 g. per day resulted in an output of 0.3 g. per day; calcium balance was still maintained.

Figure 5.2 illustrates the explanation offered. Subjects on a high calcium intake have apparently formed labile calcium deposits which are retained only as long as the high calcium intake is maintained. In Fig. 5.2 the size of the stream of water required to keep the sieve at a given level depends entirely upon what level we have decided to hold. There is no clear evidence that a maximal calcium deposition represents a biological advantage. This behavior is one that might be expected a priori from distributional considerations; the premise that a balance of intake and output should always be maintained in the adult, no matter what the starting point, has no inherent validity.

Malabsorption syndromes, which usually include steatorrhea, lead to poor calcium absorption perhaps because of an unavailability for absorption of calcium that has formed insoluble soaps with the fatty acids. Increased intakes of calcium and vitamin D may be necessary to secure calcium balance. Calcium absorption also bears a crude inverse relationship to the pH prevailing in the intestinal lumen.

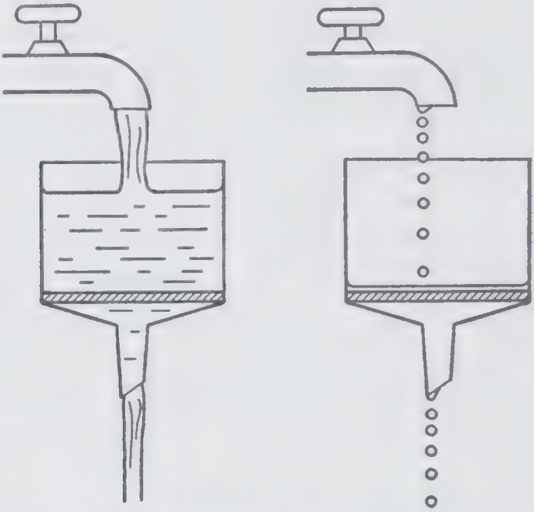

Fig. 5.2 To illustrate why a person who has had a high calcium intake may require more dietary calcium to remain in calcium balance than one who has had a low intake. Labile calcium deposits of questionable biological value (represented by the water in the sieve at the left) may require a high calcium intake for their maintenance. (After Hegsted, et al.)

Neonatal Tetany. This difficulty may also be used to illustrate the interactions of calcium and phosphate. A few years ago the use of infant dietary formulas prepared from dried milk was observed often to produce tetanic behavior. These preparations contained excessive amounts of phosphate relative to calcium. Cow's milk has relatively more phosphate to begin with, but in addition, phosphoric acid had been added to produce a smooth curd. In the adult such a high phosphate content would only have handicapped calcium absorption, but in the infant, *hyperphosphatemia* was produced, driving down the serum calcium and leading to *tetany*. As an immediate measure the calcium content of the formula was increased, thereby preventing excessive phosphate absorption (Gardner).

Table 5.1. Rickets or Osteomalacia Associated with Defects of Tubule Function

(and sometimes with other metabolic defects).

(After C. E. Dent, *J. Bone Joint Surg.*, **34B**, 266 [1952])

TYPE	TUBULE REABSORPTION DEFECT					TUBULE FUNCTION DEFECT		NAMES USED IN LITERATURE TO DESCRIBE THESE SYNDROMES
1	Phosphate							Resistant (or refractory) rickets, raised late rickets, resistance to vitamin D (RRD), essential hypophosphataemic rickets, idiopathic osteomalacia, Milkman's syndrome
2	Phosphate	Glucose						
3	Phosphate	Glucose	Amino-acids	Water *				Fanconi syndrome, Debre-de Toni-Fanconi syndrome, Lignac's disease, cystine rickets, cystinosis, rickets with cystinuria, amindiabetes, diabetes aminicus et acidaminicus, "renal rickets,"(?) hepatic rickets, hypophosphataemic glycosuric rickets
4	Phosphate	Glucose	Amino-acids	Water *	Potassium *	Urine acidification		
5	Phosphate				Potassium *	Urine acidification		Nephrocalcinosis with renal rickets and dwarfism, hyperchloraemic nephrocalcinosis, Butler-Albright syndrome, renal tubular acidosis
6	Phosphate				Potassium *	Urine acidification	Ammonia formation	

* Defect not always conspicuous.

Renal Tubular Deficiencies. Table 5.1 presents a summary of a number of patterns of renal tubular deficiencies that influence calcium and phosphorus metabolism. The wasting of phosphate is reported for all these; frequently the ability to excrete the hydrogen ion by one or both of the principal mechanisms (see Chap. 8) is also limited, the ability to concentrate hydrogen ions from the plasma into the urine in exchange for Na^+ cations being most frequently handicapped. In one type the ability to form ammonium ion is also defective. Wastage of glucose and of amino acids may also occur but it is probably of minor consequence. Faulty mineralization with dwarfism in childhood and pathologic fractures result. Probably the chronic acidosis, by decreasing the fraction of the inorganic phosphate that is in the form entering bone, causes the difficulty. Losses of Na^+ and K^+ may also be troublesome because of the inverse relationship between H^+ and alkali-metal excretion.

The aberration of calcium metabolism in the uremic stage of nephritis is of a different character, although acidosis is likely to be included. A phosphate retention elevates the serum phosphate level, sometimes as high as 20 mg. per 100 ml., driving down the level of ionized (and therefore, of course, also of un-ionized) serum calcium. Secondary hyperparathyroidism may follow as a result of the stimulus that hypocalcemia provides to the parathyroids.

LIBRARY PROBLEMS

1. Examine case 40361, *New England J. Med.*, **251**, 442 (1954), for an illustration of the use of ethylenediamine tetraacetate (EDTA or Versene) to remove excess calcium from the organism. Look up the structure of the chelate (for example, in Calvin and Martel, *The Chemistry of Chelate Compounds*, 1954) and weigh the statement that a gram of EDTA can cause the excretion of 100 g. of calcium. Consider the difficulties involved in the analysis of a serum sample that may contain the EDTA-Ca chelate: Will the ordinary oxalate precipitation methods include all the serum calcium? Should an ashing procedure be used? Do we want to include the calcium bound by EDTA in our total serum calcium determination? What technique could be used to stimulate the excretion of metals that chelate more tightly with EDTA than calcium, without disturbing the calcium

metabolism? Why had the massive doses of vitamin D been given in this case?

2. Look at cases 42221, *New England J. Med.*, **254,** 1035 (1956); 42352, *ibid.*, **255,** 241 (1956); and 42332, *ibid.*, **255,** 349 (1956). Decide which of the serum calcium levels reveal clear-cut depression of the level of *ionized* calcium. In the second case calcium-soap formation in the intestine was proposed as an explanation for the hypocalcemia. Does this seem plausible?
3. Find (a) the earliest and (b) the most recent report you can of fatal calcinosis produced by "Ertron" (vitamin D) therapy for rheumatoid arthritis. Discuss the course of events in this therapy.
4. Strontium is a member of the same periodic family as calcium and has similar properties. Which isotope of strontium is a conspicuous radioactive hazard in atom-bomb fall-out? Why is it dangerous? Is the Ca/Sr ratio increased or decreased upon incorporation of food material containing both, for example, into milk and bone?
5. Normally about one-third of the calcium leaving the body is excreted in the urine and two-thirds in the feces. Is all that in the feces derived directly from dietary calcium? The proportion excreted by the two routes may be reversed by lowering the pH of the body fluids. If the calcium excreted by the gut is in the form $Ca_3(PO_4)_2$, would a decrease in the quantity excreted by the intestine and an increase in the quantity excreted by the urine have an effect on the acid-base balance? If excess parathyroid hormone causes bone dissolution with excretion of its mineral constituents into the urine, should there tend to be an effect on the acid-base balance?
6. Examine case 43162, *New England J. Med.*, **256,** 755 (1957). Trace the argument followed in deciding whether the renal or the parathyroid disease was primary. What is the significance of the high alkaline phosphatase activity of the serum? On what basis did the discusser decide against Ca and P determinations on every patient with gastric ulcer? Comment on the administration of calcium salts to the patient post-operatively. If the sole action of the parathyroid hormone were to cause phosphate diuresis, could the formation of metastatic calcifications be readily explained?
7. Examine case 42441, *New England J. Med.*, **255,** 863 (1956). How has the disease been produced? What other diagnosis would ordinarily be suggested by the serum calcium levels found? How did the normal CO_2 content aid the discusser in reaching his diagnosis? Why should the ingested radioactive calcium have been removed from the blood more quickly than usual, as noted?
8. Examine case 37422, *New England J. Med.*, **245,** 618 (1951). Why is the serum calcium level depressed? What caused the twitchings and convulsions late in the disease? How would the secondary hyperpara-

thyroidism predicted by the discusser be produced? Can you estimate the serum ionized calcium from the data supplied?

9. Find and report researches showing that wasting and demineralization of bone result from immobilization of patients. In case 44251, *New England J. Med.*, **258**, 1259 (1958), why was an accelerated calcium excretion expected in a bedridden patient? Why was the serum Ca considered normal at 8 mg. per 100 ml? Why was the alkaline phosphatase considered surprisingly low?
10. Find a paper showing that chondroitin sulfate binds not only Ca++ but also Na+ and K+. Can you determine from this paper whether Na+, K+ or Ca++ would occupy the bound positions on this polyelectrolyte at the concentrations of these ions prevailing in the extracellular fluid?
11. A case is discussed in the *New England J. Med.*, **258**, 1053 (1958) of pancreatitis believed to have been secondary to hyperparathyroidism. Discuss (a) the high serum amylase level (Can you cite the normal range?); (b) why the serum calcium was determined; (c) the interrelationships between the serum Ca and P levels reported.

6

GAS TRANSPORT

Form of Transport. When a gas is brought into equilibrium with a solution, a certain number of gas molecules enter the solution, this number increasing with the number of molecules per unit volume of the gas phase, i.e., with the pressure of the gas. As discussed earlier, we can logically say that the gas is then at this given pressure *in the solution,* whether the separate gas phase remains present or not.

Oxygen is transferred to our cells by way of the lungs and blood stream because of the *gradient of oxygen pressures* along the course of its transfer, as illustrated in Fig. 6.1. Oxygen is not ordi-

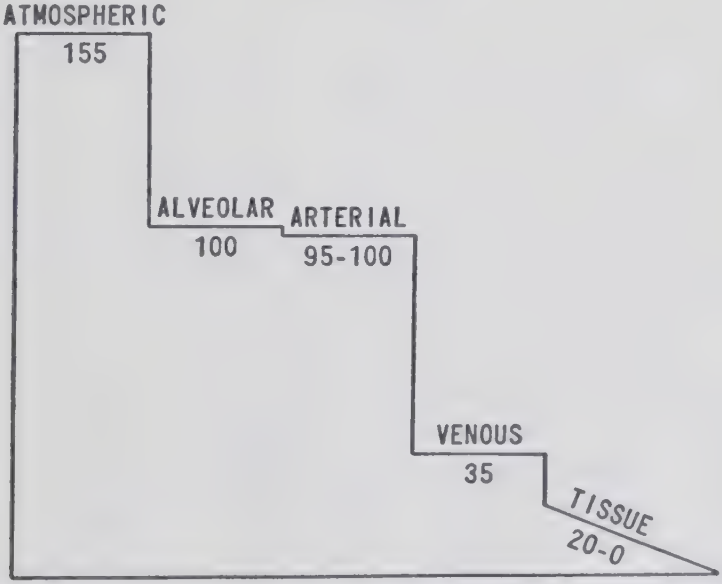

Fig. 6.1 **Gradients of oxygen pressure are utilized in oxygen transport.** The numbers are approximate oxygen pressures in millimeters of mercury.

narily pumped against a gradient, although in the case of the swim-bladders of deep-sea fish, it must be. Our tissues require oxygen only at a very low pressure (which is not accurately known) in order to convert to H_2O the hydrogen atoms transferred from foodstuffs. Most of the 155-mm. oxygen pressure of the atmosphere can be dissipated in the transfer and still leave an adequate pressure to drive these reactions.

First of all the oxygen of the atmospheric air is diluted in the pulmonary alveoli by excretory CO_2 and water vapor and by the loss of oxygen. The normal membranes are thin enough and the surface great enough so that the movement across the alveolar membranes into the blood occurs with a further, very small pressure drop. Taking the arterial blood P_{O_2} as 100 mm., the amount dissolved, as shown earlier, is given by the solubility coefficient $\alpha = 0.024$ ml. of O_2 dissolved by 1 ml. blood at $P_{O_2} = 1$ atm. At a pressure of 100/760 atm. the amount is $100/760 \times 0.024 \times 1000 = 3.2$ ml. O_2 per liter.

Now the P_{O_2} of the mixed venous blood returning to the heart at rest is about 35 mm., which corresponds to a concentration of $35/760 \times 0.024 \times 1000 = 1.1$ ml. O_2 per liter. The arterio-venous difference is 2.4 ml. O_2, which is the amount of oxygen that has been lost *from physical solution* by the passage of an average liter of blood through the capillaries.

Actually we use approximately 300 l. of oxygen per day, or 250 ml. per minute at rest. If a liter of blood can deliver only 2.4 ml., then 250/2.4, or over 100 liters of blood would need to leave the heart every minute. This is over 25 times the actual cardiac output.

The lesson of this calculation is that most of the oxygen is *not* carried in physical solution. A similar calculation can be made for CO_2 transport, using the values of 40 and 46, respectively, for the P_{CO_2} of the blood in the arteries and for the mixed venous blood. In that case we calculate the amounts dissolved to be 29.0 and 33.3 ml. per liter. The difference of 4.3 ml. CO_2 per liter of blood is much too small to account for the actual amount of CO_2 delivered by a liter of blood.

In both cases it is clear that the gases are carried mainly in modified forms.

Hemoglobin and Oxygen. Hemoglobin is a protein of molecular weight about 66,800 containing four *heme* groups (see Fig. 6.5).

Back in the days when the molecular weight of hemoglobin was known to be only about 16,700 or some multiple of this number, the symbol Hb was chosen to represent 16,700 g. of hemoglobin, the amount capable of binding one mole of O_2 (now re-estimated at 16,520 g.). Following this terminology to its logical conclusion requires us to represent the whole hemoglobin molecule as Hb_4. Heme is an iron-porphyrin in which the iron is in the ferrous state. When the iron becomes ferric, heme becomes *hematin* and hemoglobin becomes *methemoglobin* (or hem*i*globin) and no longer functional in oxygen transport. Enzyme systems of the erythrocyte operate to return methemoglobin to the hemoglobin form, and methemoglobinemia is as likely to be caused by agents that poison these enzyme systems, as by oxidizing agents. In congenital methemoglobinemia a flavoprotein reductase, needed for reduction of methemoglobin by reduced diphosphopyridine nucleotide, appears to be present in deficient quantities (Gibson). Of the red cell pigment, 10% to 45% may be in the methemoglobin form in this disease.

The hemoglobin content of blood is usually stated as the grams of the protein per 100 ml. of blood. One hundred grams of red cells contain the astonishing amount of 30 g. of hemoglobin, equivalent to 33 g. per 100 ml. of red blood cells. This latter value for the *mean corpuscular hemoglobin concentration* is, of course, subject to variation in disease. If we take a hematocrit of 46 as average, we have 46 ml., or 50 g., of red blood cells per 100 ml. of blood, and hence 15 g. of hemoglobin. For emphasis we often say, "The patient has 15 *grams* of hemoglobin," when of course we mean 15 *grams per cent*, or simply 15% hemoglobin in his blood. The latter form of statement conceivably could, however, be misunderstood to represent the ambiguous and objectionable *percentage of normal hemoglobin.*

Let us also, for comparison, express this concentration in millimoles of Hb per liter. Since a mole of Hb represents 16,500 g., a millimole is 16.5 g. Therefore 15.0 g. per 100 ml. equal $15.0/16.5 = 0.9$ millimoles per 100 ml., or 9 millimoles per liter. In the same way we can describe the normal oxygen capacity of the hemoglobin of blood as 9 millimoles per liter. In volumes per cent this is $9 \times 22.4 \div 10$, or 20 vol. per cent. That is, hemoglobin permits normal blood to take up at saturation one-fifth of its volume of oxygen gas.

As O_2 passes through the alveolar membrane into solution in the blood, the molecules in their thermal motion collide with reduced hemoglobin molecules and combine. The product, however, has a tendency to fly apart again. All we can do to describe this situation is to show how large a pressure of O_2 is required to produce a given degree of loading or saturation of the hemoglobin (Fig. 6.2). The result is a very curious curve that makes no simple mathematical sense. At first the uptake increases slowly with oxygen pressure, then more rapidly, and finally more slowly. This curious skewed curve has given rise to the term *the skew chemistry of hemoglobin.*

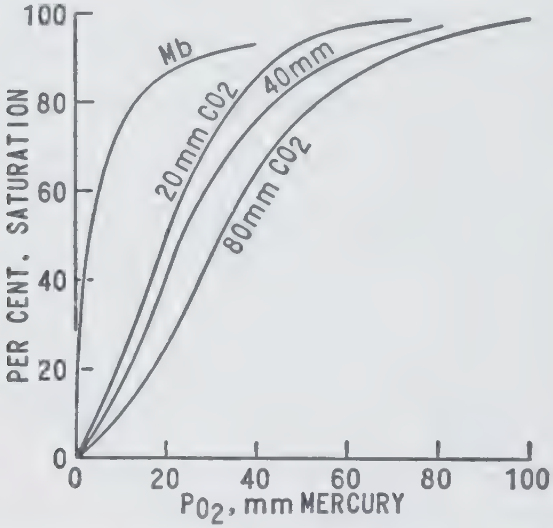

Fig. 6.2 O_2 absorption curves for blood. The three curves at the right show the absorption curves for blood at three values of the carbon dioxide pressure. The myoglobin curve is a rectangular hyperbola as illustrated at the left.

Mathematically, the relationship one would expect on applying the mass-action law to the simple reaction

$$Hb + O_2 \rightleftharpoons HbO_2$$

is a rectangular hyperbola, as seen at the left of Fig. 6.2. This in fact is the kind of curve that myoglobin yields. Myoglobin has only one heme per molecule; therefore we may assume that the skewed shape for hemoglobin arises from interactions among the four hemes when they are on one molecule. Pauling and Coryell have achieved a description of these interactions to explain the skewed curve.

In any event this oxygen absorption curve, showing as it does loading and unloading in a pressure range midway between that of the atmosphere and the oxygen pressure necessary for oxidations in our cells, is highly suitable both as to position and shape for life at reasonable altitudes on the surface of this earth. Organisms that live at low oxygen pressures possess respiratory pigments which are suitable for their environments.

Intuitively we may feel that oxygen should move from lung to tissue because that is the direction it needs to move. If, however, we stop to think about it, we realize that HbO_2 dissociation occurs in the lungs as well as in the tissues, and only the higher pressure of O_2 in the lungs causes *association* to be faster than *dissociation*. Undoubtedly many oxygen molecules are transported in the "wrong" direction by Hb from our remotest cell to the lungs!

The reaction of hemoglobin with oxygen shows a sensitivity to pH, its tendency to bind O_2 being decreased as the pH falls. In Fig. 6.2 a lower pH has been produced by repeating the study at a higher CO_2 pressure, resulting in the curve at the right. The pH of the blood falls slightly during the passage through tissue capillaries (except in the lungs) because of the rising CO_2 pressure. A slightly increased *unloading* of oxygen results. This effect will be discussed further in connection with CO_2 transport.

Incidentally, this process *could* operate as a pumping mechanism. That is, if the pH difference were large, the oxygen could be unloaded at a pressure *higher* than the one at which it had been loaded. This operation has been proposed to explain the pumping of oxygen into the swim-bladders of fish. Hemoglobin is actually an interesting example of a substance that could operate as a carrier for active transport, although in our organism it is not known to do so.[1]

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[1] This hypothetical case of active transport by hemoglobin can serve to illustrate the misleading phenomenon encountered when labels are used to observe flux, namely, *exchange diffusion*. Suppose, because of a local pH fall, Hb releases O_2 into the swim-bladder of a fish at a pressure higher than that prevail-

One would not suppose, for a molecule of the size of hemoglobin containing over 500 amino-acid residues, that every detail of the molecular structure would be critical. Remarkably, however, a genetic variation in the globin molecule by which *only a single amino-acid residue* is aberrant causes the severe hereditary *sickle-cell disease.* According to Ingram a valine residue (five carbon atoms) is substituted for a glutamic acid residue (also five carbon atoms). This change causes a shift of the oxygen absorption curve observed *in vivo* to the right, so that higher oxygen pressures are necessary to produce a given degree of saturation (Rodman). The charge on the protein molecule, and hence its electrophoretic behavior, is materially changed. A dangerous aspect is that at lowered O_2 pressures the sickle-cell hemoglobin enters pseudo-crystalline aggregates in the erythrocyte, so that the red cell is readily destroyed. Thrombosis and embolism, rather than anemia, are the dangerous consequences. This disease is a classical example of *molecular disease.*

Forms of Carbon Dioxide Transported. Handling CO_2 introduces several complexities that do not occur in oxygen transport. Two reactions dominate the account:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \tag{6.1}$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \tag{6.2}$$

If these reactions are understood, the subject is comparatively simple. By these reactions we get one H^+ for almost every CO_2

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ing in the surrounding sea water. Now if a trace of labeled O_2 is introduced into the sea water, the O_2 will combine reversibly with Hb in the gills. Upon reaching the swim-bladder, the HbO_2 will rapidly dissociate. The Hb formed, however, will almost as rapidly recombine with the free oxygen (mostly unlabeled) at that site, and much of the hemoglobin will return to the gills as HbO_2. You will understand that the rate of exchange of the labeled O_2 of the sea water with O_2 of the swim-bladder cannot serve to measure the rate of active transport. The idle one-for-one exchange represented by the wasteful wrong-way transport, together with the amount of right-way transport which it cancels out, illustrates what is meant by *exchange diffusion.* In short, exchange diffusion is the error in assuming that the carrier-bound flux is all active transport. (Reference may be made to Fig. 4.2 in connection with this footnote, substituting O_2 for Na^+ and Hb for C, and considering that *out* represents the interior of the swim-bladder.)

molecule formed by the oxidation of foodstuffs. In a day this amounts to 13 to 20 *moles* of CO_2. The amount of H^+ contributed is equivalent to that present in a very impressive quantity of strong mineral acid. Of course only an exceedingly small part of this H^+ is actually free at any one instant, it being removed from the body as quickly as it is formed and most of it being bound to various buffer anions during its transient existence.

Earlier in this chapter we calculated that the physical dissolving of CO_2 (including whatever quantity of carbonic acid is formed by reaction 6.1) can account for only a minor fraction of the CO_2 actually transported. We are dependent upon reaction 6.2 for the transport of most of the CO_2.

The actual amount of CO_2 found in various forms in whole blood is illustrated by the following classical analysis (Dill, as calculated by Hastings):

FORM	ARTERIAL IN M./L.	VENOUS IN M./L.	DIFFERENCE IN M./L.	% OF TOTAL CARRIED
Total CO_2	22.0	24.2	2.2	100
Free H_2CO_3	1.3	1.5	0.2	9
Bicarbonate ion	19.6	21.0	1.4	64
As $HbNHCOO^-$	1.1	1.7	0.6	27

As the table shows, whether we analyze venous or arterial blood, we find rather high total CO_2 contents. This CO_2 is present in three forms: physically dissolved ($CO_2 + H_2CO_3$), the bicarbonate ion, and an unstable combination with hemoglobin, the carbamino-CO_2.[2] Of the total CO_2 only about 10% in venous blood is unloaded in the lungs, about 2.2 millimoles per liter in the case illustrated. The important question is: What fraction of this difference is accounted for by the various forms of CO_2?

Physically dissolved carbon dioxide ($CO_2 + H_2CO_3$) increases by about 0.2 millimole per liter under the change of P_{CO_2} from 40 to 46 mm. from the arterial to the venous side of the circulation, representing about 9% of the total loading of CO_2. The catalysis of the hydration, reaction 6.1, in either direction by carbonic anhydrase is an important factor.

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[2] It should be emphasized that the amount of CO_2 carried in this form has been estimated indirectly so that a number of possible errors are collected.

The second form of CO_2, bicarbonate ion, is formed by reaction 6.2. The important feature is that *a hydrogen ion* is released for every bicarbonate ion formed. As the foregoing table shows, about 1.4 millimoles of H^+ must be added to each liter of blood by this reaction when it receives its new CO_2 load in the capillaries. One millimole of H^+ per liter (0.001M) is enough to bring the pH of pure water to 3.0. We may temporarily imagine, as illustrated in the extreme left-hand portion of Fig. 6.3, that the pH of the blood would oscillate between 7.4 and 3 as it passes from the arterial to the venous state. Of course this is absurd because reaction 6.2 would be blocked quickly if there actually were such an accumulation of hydrogen ions. Nevertheless this quantity of H^+ must be disposed

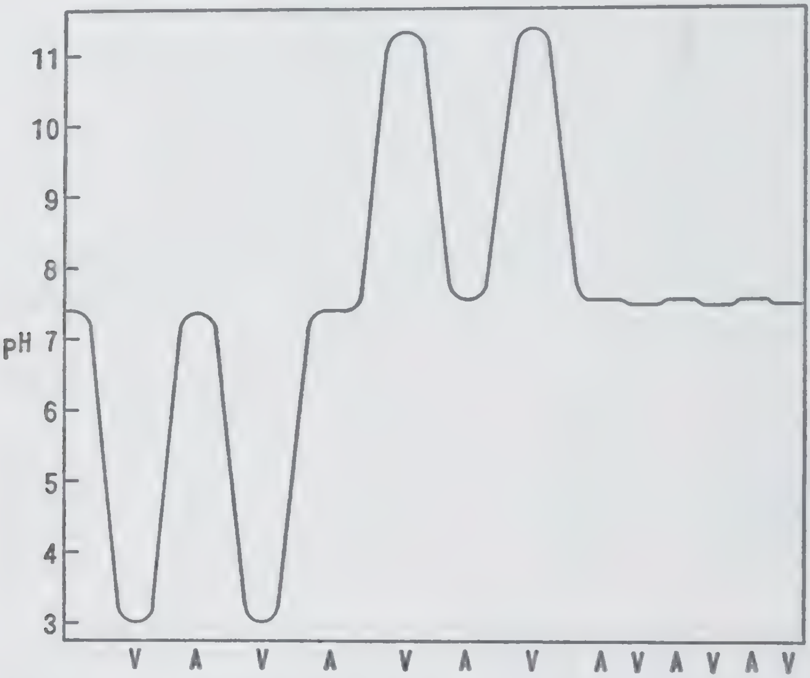


Fig. 6.3 Imaginary visualization of the sweeps in pH produced from arterial to venous blood (on the left) if CO_2 transport occurred alone, or if (in the middle) O_2 transport occurred alone. In actuality, of course, the consequences would be that much less CO_2 and O_2 would be taken up, each uptake being blocked by the pH change it brought about. At the extreme right is shown the extent to which the two opposite influences almost cancel out in the real situation.

of to permit reaction 6.2 to proceed far enough and with safety. This is the main problem of CO_2 transport.

Our first thought is that the *buffering* properties of blood will take up most of this H^+. Now the driving force that causes buffer anions to take up more H^+ is the *increased concentration* of H^+. In other words the pH must actually be lowered to occasion buffering, and if we graph the appropriate portion of the titration curve of whole blood, we can discover how much H^+ uptake will arise from a given increase in H^+ concentration (Fig. 6.4). Most of the buffering is actually due to hemoglobin, specifically to the imidazole groups of the histidine residues contained in it.

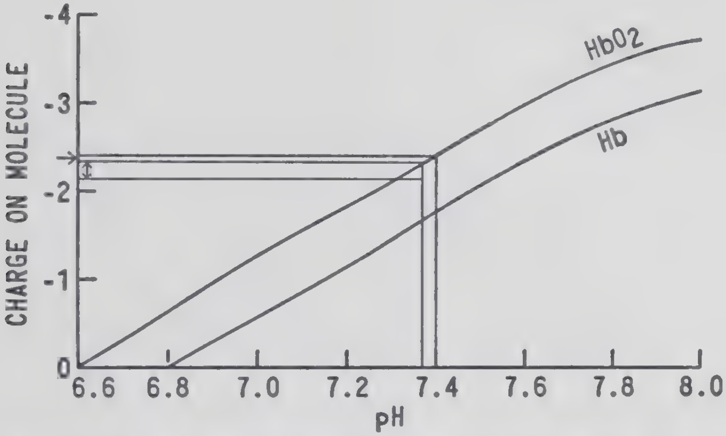


Fig. 6.4 Portions of the titration curves of oxygenated and reduced hemoglobin. The scale at the left shows the net negative charge on the molecules. Measuring downward, one sees the quantity of hydrogen ions taken up. It may be seen that the fall in pH from 7.40 to 7.37 will permit the uptake of the small amount of hydrogen ion indicated by the arrow at the left if the HbO_2 remains oxygenated. But if at the same time 30% of the HbO_2 is reduced, the additional amount of H^+, indicated by the symbol ↕ , can be taken up. The latter quantity is taken up without benefit of pH change, i.e., *isohydrically*. Note that this illustration also shows that one cannot accurately represent Hb as a monobasic acid; for example, HHb. HbO_2 is shown as isoelectric (no net charge) at pH 6.6, Hb at pH 6.8.

The amount of hydrogen ion taken up by this buffering, however, is in fact minimized by the smallness of the pH fall that actually occurs. At rest the pH difference between arterial and venous blood appears to be only about 0.03 unit (for example, 7.41 vs. 7.38). In other words some other factor is so successful in taking up the released hydrogen ions that what we ordinarily understand as buffering remains a minor factor.

As the blood is gaining CO_2 in passing through the capillaries, however, another change is occurring. Oxygen is diffusing from the blood; about one-fourth to one-third of the oxyhemoglobin is passing to the reduced (nonoxygen bearing) form. This is a new chemical substance, and its titration curve has a distinctly different position in this range (Fig. 6.4) than oxyhemoglobin. The reduced form has a greater tendency to bind the hydrogen ion; the imidazole groups representing this portion of the titration curve are more weakly acidic. Hence, in Fig. 6.4, far more hydrogen ions are taken up by this conversion than by buffering proper. Since this effect may be described as occurring at constant pH and is not dependent on the pH, it is called *isohydric;* for example, the *isohydric transport* of CO_2.

Let us reconsider this behavior. Suppose you had in a test tube a weak acid HA in equilibrium with its dissociation products. Suppose you could change this system at will into another *weaker* acid system HC,

$$\begin{array}{l} HC \rightleftharpoons H^+ + C^- \\ \downarrow\uparrow \\ HA \rightleftharpoons H^+ + A^- \end{array}$$

As you made the conversion from HC to HA you would cause a sudden release of H^+ by HA because of its greater tendency to dissociate. The conversion in the other direction would cause alkalinization. This is the way in which hydrogen ions are released and taken up as hemoglobin takes up and releases oxygen.

(One oversimplification is to write HHb for hemoglobin, illustrating only the hydrogen ion that tends to be added when oxygen is lost. If we use this shorthand we must meanwhile remember that oxyhemoglobin and reduced hemoglobin are, like all proteins, *polybasic* ampholytes, as Fig. 6.4 illustrates.)

We can imagine, as shown in Fig. 6.3, that the transport of *oxygen alone* would occasion wild sweeps of the pH upward and downward. Here again the uptake of oxygen actually would be seriously limited by the hydrogen ion released if the H+ were not disposed of, so that the actual pH changes would not be so large. We may then visualize that when O_2 and CO_2 transport are occurring together (in opposite directions), the two oscillations of hydrogen-ion concentration will almost balance out as shown, limiting the pH change to about 0.03 units.

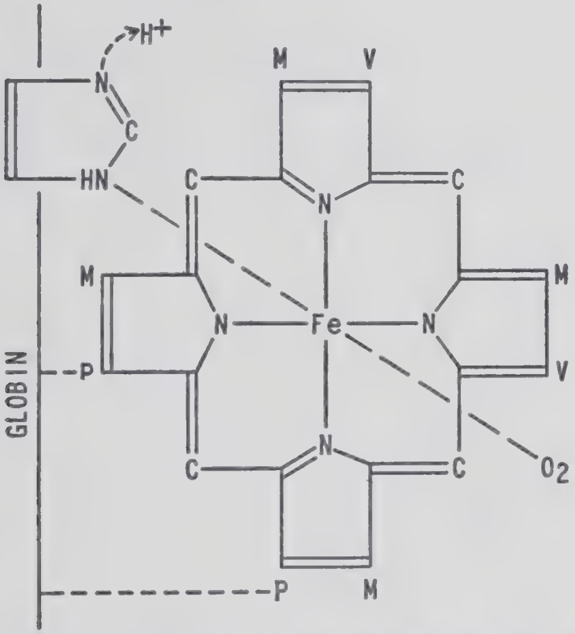


Fig. 6.5 The six coordination bonds of FeII in HbO$_2$. M = a methyl group, V = a vinyl group, P = a propionate, which links to the globin part of the molecule. An imidazole group belonging to a histidine residue in globin serves as the fifth point of coordination. When an O_2 molecule is bound at the sixth position (lower right), the resulting shift in electron positions has a strong tendency to cause the H+ to dissociate from the imidazole group. Conversely, when this H+ is forced on by a pH fall, the O_2 has a tendency to be lost.

Why Does Hemoglobin Tend to Release H+ when It Binds Oxygen? Four of the six coordination positions of the iron of hemoglobin are occupied by pyrrole nitrogen atoms (Fig. 6.5). The fifth is occupied by an imidazole residue of a histidine bound in a peptide chain of globin; the sixth binds O_2. Probably when oxygen is absent, the sixth position is coordinated to a second imidazole group.

Let us focus our attention on the axis passing through the iron atom from O_2 to an imidazole group. The addition of the oxygen molecule causes a shift in the position of electrons at the imidazole group which weakens its hold on a hydrogen ion. Perhaps for analogy we can think of a mechanical device arranged so that placing an O_2 on one end of the axis will cause the H^+ to fly off the other end. Conversely, adding a H^+ to the imidazole end will tend to displace the oxygen from its position (which reveals why, as shown earlier in Fig. 6.2, the oxygen absorption curve is displaced by a pH change). Our mechanical picture must be corrected somewhat because adding the oxygen merely creates a *tendency* to cause the H^+ to dissociate: About 0.7 equivalents of H^+ have been observed to be released when one mole of O_2 is added.

The formation of carbamino-CO_2 is analogous to bicarbonate formation:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

$$CO_2 + HbNH_2 \rightleftharpoons HbNHCOO^- + H^+$$

(The NH_2 group shown is part of the globin molecule.) Each reaction forms H^+, which must be disposed of by the processes just described. This new reaction, to whatever degree it exists, increases the total CO_2 transported under a given pressure gradient, but it does not minimize the principal problem of *hydrogen-ion disposition.*

Figures 6.6 and 6.7 represent a recapitulation of the above sequence of events and should be imagined as parts of an animated model. In Fig. 6.6 *oxygen transport* is occurring alone, i.e., at constant CO_2 pressure. Ten molecules of Hb are visualized moving through the circulation. In the right-hand (venous) limb, seven of the ten bear oxygen, three do not. As they enter the pulmonary capillaries these remaining three gain oxygen and release two hydrogen ions, making the blood acid. At the lower end three oxygen molecules are again released because of the lower prevailing P_{O_2}, and the two hydrogen ions are taken up again. The pH oscillates strongly from acid to alkaline.

In Fig. 6.7, CO_2 transport is pictured alone, i.e., at constant P_{O_2}. At the bottom in the nonpulmonary capillaries carbon dioxide enters the blood and causes the release of, let us say, two hydrogen ions, thus causing the "venous" blood to become acid. At the lungs these hydrogen ions are taken up again as the gained CO_2 is released. Again we have a strongly oscillating pH, but in the opposite sense.

If Fig. 6.6 is laid over Fig. 6.7 as in Fig. 6.8, we can visualize that the hydrogen ions released by the incoming CO_2 are taken up by the hemoglobin, which has just lost its oxygen and become receptive to H^+; at the top (pulmonary) end the hydrogen ions released as the reduced hemoglobins gain oxygen are exactly what are needed to permit the bicarbonate to form CO_2 again so that this can escape.

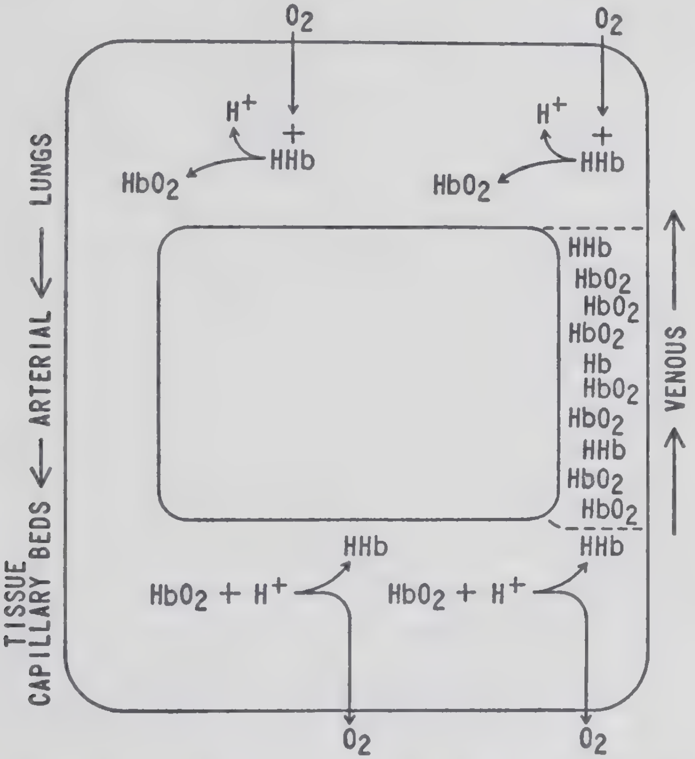


Fig. 6.6 Visualization of blood oxygen transport occurring alone. Hydrogen ions are released in the lungs and bound again in the nonpulmonary tissues, causing large pH changes and serious interference with oxygen uptake and release. (For technical reasons only two of the three Hb molecules mentioned in the text are shown reacting with oxygen.)

We may in summary look upon gas transport as a juggling back and forth of the hydrogen ion between hemoglobin and the bicarbonate ion, each acting as the acceptor when the other must be the donor. This interlocking permits the uptake of each gas to be much more extensive for a given increase of its partial pressure; looked at in another way, it permits both to be transported with minimal changes in pH. Exactly how closely the hydrogen ions released by one suffice to be bound by the other depends somewhat upon the relationship between oxygen consumption and CO_2 release, i.e., upon the respiratory quotient. Do normal values for the human respiratory quotient permit maximal pH stability?

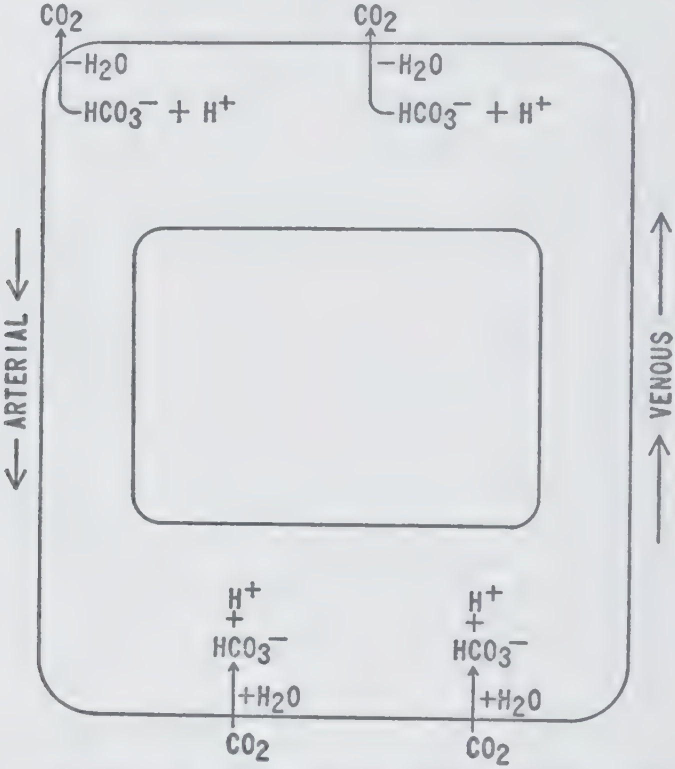


Fig. 6.7 Visualization of blood carbon dioxide transport occurring alone. Hydrogen ions are released at the nonpulmonary tissues and bound again in the lungs, producing large pH changes and prohibitive interference with CO_2 uptake and release. If Fig. 6.6 is laid over Fig. 6.7, one can see that the hydrogen ions released by one process are nearly all consumed by the other when O_2 and CO_2 transport are both occurring, but in opposite directions.

Heterogeneity of Blood. So far we have treated blood as if it were a single *homogenous* solution, ignoring the fact that the hemoglobin is enclosed in minute sacs. The consequences are merely an additional complication but no apparent loss of efficiency. Of course if the hemoglobin were free in the plasma, it would be exposed to undue urinary loss. Small amounts of hemoglobin in plasma (up to about 130 mg. per 100 ml.) become bound to another protein and do not pass the glomerulus. When the levels exceed the binding capacity of this protein, hemoglobinuria ensues (Lathem).

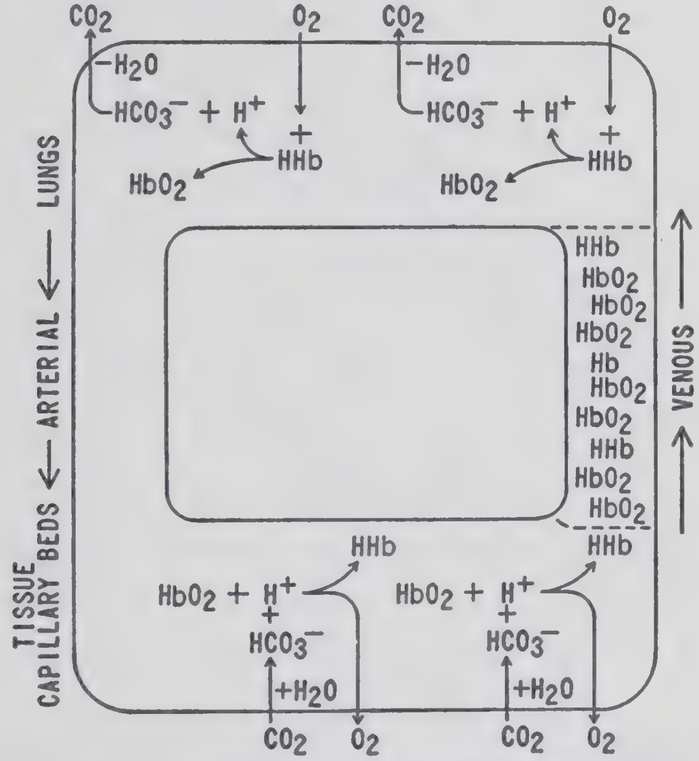


Fig. 6.8 Visualization of oxygen and carbon dioxide transport occurring together. A combination of Figs. 6.6 and 6.7.

An arterio-venous differential of 2.2 millimoles of CO_2 per liter has been offered as typical. If we separate the cells and serum, we find about one-half of this extra carbon dioxide in each phase. Perhaps it is a paradox, if hemoglobin actually accounts for 90% of the transport of CO_2, that one-half the CO_2 "pay-load" is in the plasma.

What Hb carries for the most part is not CO_2 itself but the H^+ that the CO_2 has donated, thereby forming HCO_3^-. The resulting

HCO_3^- can move freely out of the erythrocyte, which it permeates readily, so that the plasma shares in the extra bicarbonate ion acquired. The free permeation of the bicarbonate ion could not occur if the erythrocyte were not also permeable to the chloride ion. As bicarbonate ions move in one direction, chloride moves in the other to preserve electroneutrality. (Why should not transfers of K^+ out of the cells with the HCO_3^- serve instead?)

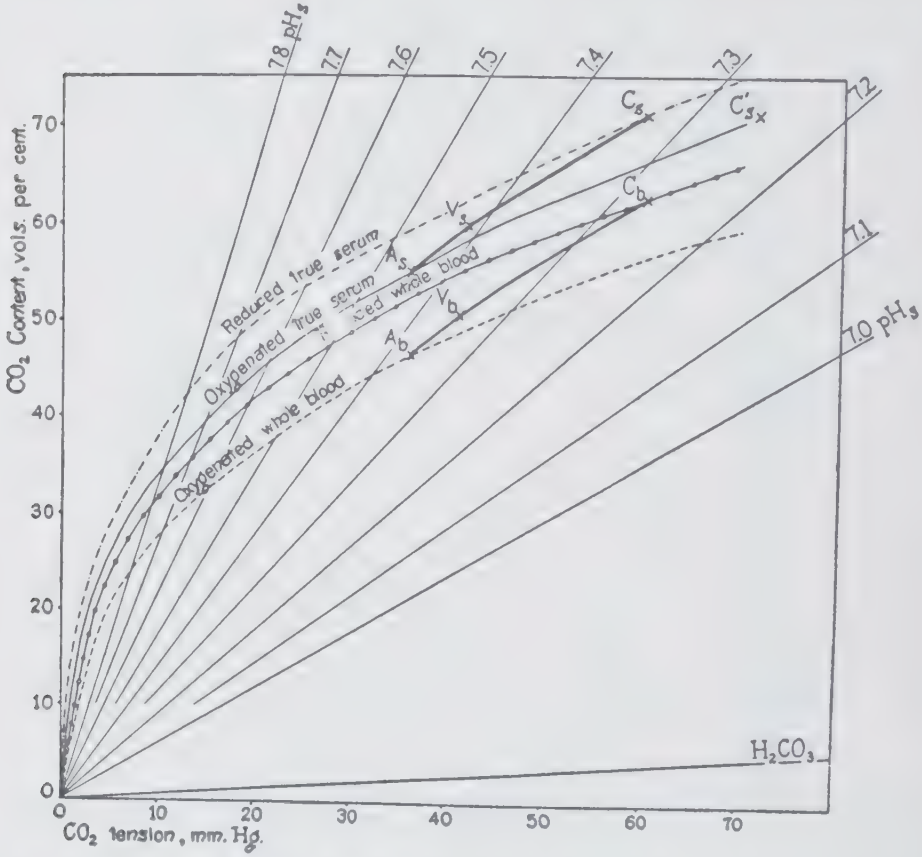


Fig. 6.9 Carbon dioxide absorption curves of blood. (From Peters and Van Slyke, *Quantitative Clinical Chemistry,* vol. I, Baltimore, 1931.)

This "chloride shift" is a complication of analytical importance. If a sample of blood is left standing exposed to the atmosphere for an extended period, almost all the CO_2 will be lost from it. This process is merely an exaggeration of the changes that take place in the lungs as CO_2 is lost. Bicarbonate ion is lost by reaction with hydrogen ions in the erythrocyte to form CO_2 and H_2O. Hence the HCO_3^- concentration within the cells becomes lower than that outside, and bicarbonate moves in to be likewise destroyed, while chloride moves out. Accordingly, subsequent analysis of the serum chloride will give a falsely high value. Therefore blood must be handled carefully for serum chloride analyses. Ideally it should be collected under oil; in any event the red cells should be separated promptly with minimal agitation. Water transfer also occurs as the blood is alkalinized by CO_2 loss from the whole blood.

CO_2 Absorption Curves. Figure 6.9 shows another popular method for representing the behavior of blood toward carbon dioxide at various degrees of oxygenation. The pressures of carbon dioxide required to produce given CO_2 contents are shown.

Water or a bicarbonate solution follows Henry's law and takes up a comparatively small amount of CO_2 (lower line). Oxygenated blood takes up a great deal more CO_2 for given CO_2 pressures. But if the hemoglobin is reduced, its affinity for H^+ is greater, and even more CO_2 is taken up at each value of the CO_2 pressure. The student should estimate the position of two points, A and V on this chart, to illustrate the arterial-venous oscillation in the CO_2 content during the respiratory cycle. Figure 6.8 will receive further development in the next chapter.

LIBRARY PROBLEMS

1. The cytochromes contain the same iron porphyrin as hemoglobin. How are these proteins different from hemoglobin (a) in the linkage of the heme to the protein, and (b) in the change of the molecule which occurs functionally?
2. Find a textbook discussion of CO_2 transport that appears to make the potassium ion extremely important to the process. Show how that discussion is related to the one given in this chapter.
3. Discuss quantitatively the reaction of hemoglobin with carbon monoxide. What convenient *physiological* method could be used for sampling the air in a vehicular tunnel to see if the average carbon monoxide content is dangerous? What color changes does a CO-poisoned person show?
4. For case 44211, *New England J. Med.*, **258**, 1054 (1958), discuss how the oxygen determinations on blood samples obtained by catheterization contributed to the clinical diagnosis.

5. Cyanide tends to combine with the iron of iron-porphyrins. Considering that a very small dose of cyanide can be fatal, do you think it is acting by combining with hemoglogin? In general what conclusion can you draw as to the site of action of any substance, small doses of which produce a powerful pharmacologic action?

7

RESPIRATORY INFLUENCES ON HYDROGEN-ION DISTRIBUTION

A substantial part of this subject has in fact already been covered. The transport of either CO_2 or O_2 causes the transient release of substantial quantities of hydrogen ion. Even though these releases are transient, they would be dangerous if they did not almost cancel each other, the bicarbonate ion acting as a H^+ acceptor when hemoglobin must be a H^+ donor, and vice versa.

The exacerbations of the problems of gas transport, especially CO_2 accumulation and CO_2 overexcretion, constitute the *respiratory* disturbances of the acid-base balance. When the excretion of CO_2 is retarded by disease, the transient load of hydrogen ions becomes abnormally large, causing respiratory acidosis; conversely, if excessive stimulation of the breathing causes CO_2 to be excreted faster than it is formed, the body fluids suffer a net loss of hydrogen ions and *respiratory alkalosis* results. Against these respiratory disturbances hemoglobin is the principal blood buffer, just as it is during gas transport.

Metabolic Disturbances of Hydrogen-Ion Concentration. The situation is very different in the second, more frequent type of disturbance of neutrality, the so-called *metabolic* acidosis or alkalosis. A metabolic acidosis is one caused by an acid stronger than carbonic acid. We shall discuss in a later chapter more plausible sources of acidosis. However, to illustrate this situation, let us suppose that hydrochloric acid is injected in a near-maximal quantity into an animal. Such an experiment found the portion of the injected H^+ remaining in the blood distributed as follows:

Neutralized by	*Milliequiv./l.*
HCO_3^-	18
hemoglobin	8
all other buffers	2

(A further large part of the H^+ injected will have been disposed of in the interstitial fluid and in the cells.)

Note that now we have elevated the bicarbonate ion to the top of our list of blood buffers, whereas we ignored it entirely as a defense against CO_2 accumulation. Is this an inconsistency?

Actually the bicarbonate-carbonic acid system is a very poor *buffer,* in the normal sense, at the pH of normal plasma. Note that the titration curve (Fig. 7.1) lies mainly below pH 7, and that the curve is far from steep at pH 7.4.

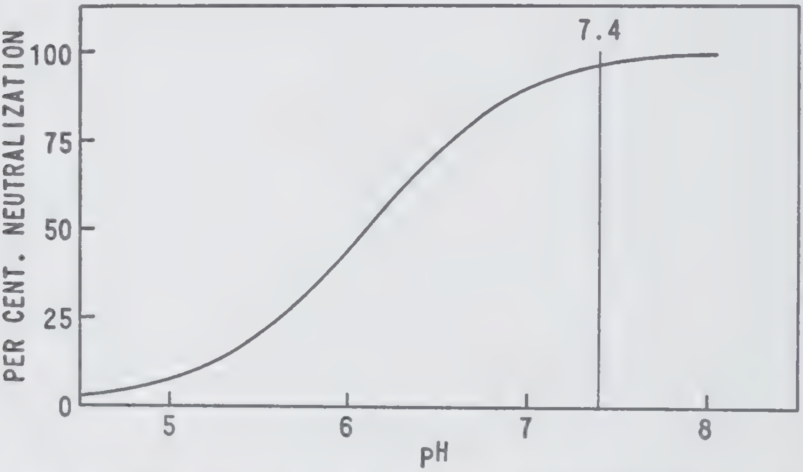


Fig. 7.1 Titration curve for the system, bicarbonate-carbonic acid. At pH 7.4 we are too far from the steep middle part of the curve to get good buffering.

The main value of HCO_3^- in getting rid of extra H^+ comes from another circumstance. The other member of the buffer system, carbonic acid, is unstable, and its concentration depends only upon the CO_2 pressure. When extra H_2CO_3 is formed by reaction of HCO_3^- with H^+, it breaks down to water and CO_2, and the latter is promptly excreted:

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow H_2O + CO_2 \uparrow$$

Ordinary buffers do not do this: When we *decrease* the concentration of the dissociated form, we *increase* the concentration of the undissociated form. But in the case of HCO_3^- we can sacrifice this ion without permanently increasing the H_2CO_3 level.

Demonstration[1] of Respiratory Response to Acid. Let us set up the following demonstration: in two closed cylinders place saline solutions prepared to mimic our extracellular fluid. One contains, among other ions, 25 milliequivalents per liter of bicarbonate ion, approximately the serum level. The other contains one-half as much bicarbonate, 12.5 milliequivalents per liter. Phenol red indicator is present in each solution.

Now let us bubble through the two solutions a mixture from a gas tank containing 5% CO_2. If the atmospheric pressure is 760 mm., the gas bubbling through the solution will have a CO_2 pressure equal to 5% of $(760 - 25)$ mm. of Hg, taking the pressure of water vapor as 25 mm. Hence the CO_2 pressure is about 37 mm. of Hg.

We notice that the two saline solutions lose their deep red color and take on two different orange hues as they come to equilibrium with this gas phase. The first solution comes to a pH of about 7.4. This in fact is the way in which we set up a pH 7.4 bicarbonate-carbonic acid buffer; we may well use the solution we have prepared as a medium for incubating various cells to study cellular processes. We can calculate with the Henderson-Hasselbalch equation that the carbonic acid concentration must be 1/20 of the bicarbonate level, or 1.25 millimolar. Our cylinder with gas bubbling through it is thus a model of our body fluids, maintained as they are under a rather high CO_2 pressure.

Recalling that for this system

$$\mathrm{pH} = 6.1 + \log \frac{[\mathrm{HCO_3}^-]}{f \cdot P_{\mathrm{CO_2}}}$$

---

[1] DEMONSTRATION PROCEDURE. 162 ml. of $0.154M$ $NaHCO_3$ plus 828 ml. $0.154M$ NaCl plus 10 ml. 0.04% phenol red. After mixing, place one-half of the total in each cylinder. To one cylinder add 6.2 ml. of N HCl to produce $[HCO_3^-] =$ 12.5 millimolar. The other cylinder is 25 millimolar. When both have come into equilibrium with the 5% CO_2, the difference in hues is demonstrated. Then 6.2 ml. of N HCl may be added to the more alkaline cylinder, gassing reinstituted, and the successive color changes considered.

what will be the pH of the second cylinder with one-half as large a bicarbonate concentration? Here we can recall one of the landmarks of our pH scale. When we halve the A^- concentration, we shall double the hydrogen-ion concentration and lower the pH by the log of 2, or by 0.3. Hence our second solution is a pH 7.1 buffer.

Now let us add to the first cylinder by pipet enough HCl to convert *one-half* the bicarbonate to carbonic acid. Now

$$\mathrm{pH} = 6.10 + \log \frac{12.5}{13.75}$$

$$\mathrm{pH} = 6.10 - 0.04 = 6.06$$

The yellow color of the solution confirms that we have indeed brought the pH below the range of the phenol red indicator. If the HCO_3^-/H_2CO_3 system were an ordinary buffer system, there would be no further change, and our model would have, so to speak, a fatal acidosis.

This new carbonic acid concentration, however, is far higher than can continue to exist at the CO_2 pressure represented by our flowing gas phase. Therefore the carbonic acid breaks down and the extra CO_2 is carried out, and in a few minutes the carbonic acid concentration returns to the original value. Now what is the pH?

You will see that we have merely halved the bicarbonate concentration without changing the carbonic acid level, and that the pH must therefore be 7.1. The first and second solutions are in fact now identical, and one sees that they assume the same hue.

(Are you baffled by our use of a *CO_2-containing gas mixture to remove CO_2 from this solution?* This is another illustration well worth study that shows us that the laws of distribution are *quantitative* laws. Notice that a similar CO_2-loaded gas mixture present in our pulmonary alveoli serves to remove *our metabolic* CO_2 from our bodies.)

Comparison of Our Demonstration Model and the Living Organism. Because of this special property of the buffer system, the pH has been returned to 7.1 instead of remaining at pH 6.1, as it would have with an ordinary buffer. Can you suggest any way in which we can bring our cylinderful of solution back to pH 7.4, except by opening the cylinder and adding NaOH or $NaHCO_3$?

What would happen if for the 5% CO_2 we should substitute another gas mixture containing 2.5% CO_2? Then we would have halved the bicarbonate concentration and halved the carbonic acid concentration; according to the Henderson-Hasselbalch equation our pH should be the original 7.4.

The living organism *does* go further than our model has gone in correcting the acidosis; in addition to excreting the new-formed carbonic acid as CO_2, the animal continues to breathe more deeply as long as the pH is below normal, thereby mixing the alveolar air more rapidly with atmospheric air and lowering the alveolar and blood CO_2 pressure. Careful studies have shown that this response does *not* tend to go so far as to bring the pH all the way back to 7.4, thus producing the textbook conception of the *completely compensated acidosis.* If the respiratory centers were to do this, it would mean that they would respond exclusively to pH and that a highly *abnormal* CO_2 pressure would be produced in order to obtain a *normal* pH. Instead, the organism responds as if it were almost equally responsive to *lowered* pH and to *lowered* CO_2 pressure, producing the compromise of a *somewhat lowered* pH and a *somewhat lowered* CO_2 pressure. Therefore an acidosis accompanied by a normal pH is not a frequent finding.

Has our experimental model been returned to a normal situation by the demonstrated responses? Could it withstand another acid injection of the same size? By measuring the bicarbonate concentration (now reduced to 12.5 millimolar), we can see what the organism retains as a remaining reserve against acid. Therefore the serum bicarbonate concentration is used to measure the reserve of defenses against acid, leading to the term *alkali reserve* for this concentration. The curious form of the term has mainly historic interest. In our model the bicarbonate concentration represents the *whole* of the reserve against acidosis; in the living organism it does not. Hemoglobin has a considerable importance, as do tissue proteins and the tissue phosphoric esters. The bicarbonate level, however, is considered to serve perhaps as an *index* of the total reserves of all sorts.

Stepwise Recapitulation. Figure 7.2 visualizes the response of an animal to an acid injection in a stepwise fashion. As in the

Henderson-Hasselbalch equation the serum bicarbonate-ion concentration is shown above the line; the carbonic acid, below. An amount of acid is injected which will change the normal ratio of perhaps 26:1.3 millimoles to one of say 13.7:13.7. By the Henderson-Hasselbalch equation the pH should be 6.1. The lower curve illustrates diagrammatically that the incoming acid strongly stimulates the respiratory rate and volume, so that the new CO_2 is quickly swept out and the pH never actually falls as low as 6.1.

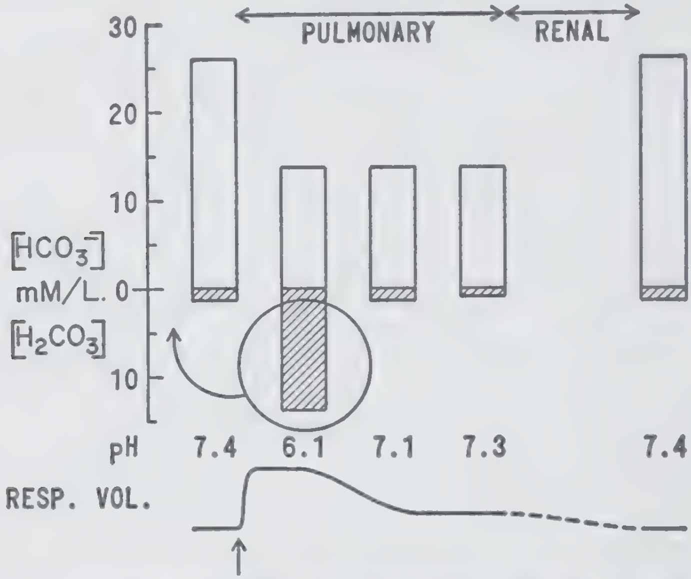


Fig. 7.2 Illustrating stepwise the pulmonary response to acid invasion (not carbonic acid). We assume enough acid is administered at the arrow to cause almost one-half the HCO_3^- to be converted to H_2CO_3. (At the same time other buffer anions also accept H+.) An explosive increase in respiration results, so that the new-formed H_2CO_3 is swept out as CO_2, and the hypothetical pH of 6.1 (second stage) is never reached. The third stage shows the transient picture when the P_{CO_2} has returned to a normal value. The respiratory rate, however, continues to be accelerated until the P_{CO_2} is brought to a subnormal point, so that the pH is brought still nearer normal. Until renal activity is able to return the bicarbonate level to normal (fifth column) the pH does not tend to be normal.

At the third stage the accelerated respiration has brought the carbonic acid concentration to normal. Since $[HCO_3^-]/[H_2CO_3]$ is now about 10, the pH is 7.1. This is as far as our demonstration model went in compensation. But the animal now continues to breathe more rapidly than normally, to bring it to stage 4, which shows the carbonic acid maintained at *subnormal* levels until the bicarbonate level is returned to normal.

Limits of Respiratory Compensation. The final stage, returning the bicarbonate concentration to normal, cannot be performed by the respiratory system. The bar diagrams of Fig. 7.3 visualize the changes in the electrolyte framework of the extracellular fluid that our injection has produced. The injected hydrogen ion has decreased the bicarbonate concentration by 12.5 milliequivalents; the injected chloride ion has taken its place. We cannot conceivably return the bicarbonate concentration to normal without excreting the extra chloride ion (relative to Na^+), which the lungs cannot do.

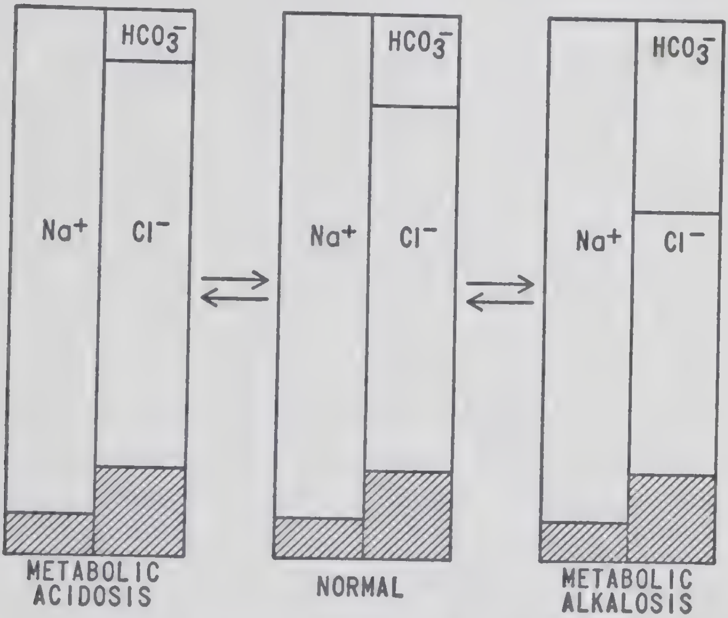


Fig. 7.3 Change in electrolyte framework after compensation of HCl or NaOH injection. In the former case chloride ion has taken the place of the destroyed HCO_3^-; in the second case the HCO_3^- generated from $OH^- + CO_2 \rightarrow HCO_3^-$ has been added to the column, along with the injected Na^+. Adjustments in water excretion minimize the change in the total height of the columns. Return to a normal picture requires renal adjustments in Na^+ and Cl^- excretion.

In actual fact neither can the lungs actually *excrete* the hydrogen ion, as will be shown in Chap. 8. The final stage of the compensation is the renal one, also discussed in Chap. 8.

Although respiration has *minimized* the pH change, the compensation is not ultimate. We have a *living* subject but one less able to withstand further similar attacks by acid. Nearly all the extracellular bicarbonate can be sacrificed before the acidosis becomes fatal. Note that the respiratory mechanism operates directly on the blood but that bicarbonate ion has been lost also from the much more voluminous interstitial fluid and perhaps also from the cells, so that the respiratory compensation is by no means limited to the blood bicarbonate. Cell buffers also play a large role in buffering hydrogen ion. Presumably the extent to which the various cells share in the buffering would be enhanced if instead of HCl we use an acid the anion of which can readily enter the cells. Otherwise the cell buffering presumably will require exchange, for example, of cell potassium for hydrogen ions.

Contrast with Respiratory Acidosis. Suppose instead that the threat is one of CO_2 accumulation. Perhaps one rebreathes the same air over and over again from a bag, or perhaps ventilation is compromised by emphysema or by open-chest surgery. Can the accumulating carbonic acid be handled like the injected HCl? The inherent character of the disturbance *prevents* any respiratory compensation. Our bicarbonate-carbonic acid buffer system is now returned to the status of a simple buffer and accordingly loses its place at the top of the list on page 98. Hence *the next* highest buffer on the list, *hemoglobin,* is at the top as far as blood-gas transport and its aberrations are concerned.

Respiratory Alkalosis. We can, by will power, breathe more rapidly than usual and lower the carbonic acid content of our body fluids. In actuality we live in a high internal CO_2 atmosphere which is arbitrarily maintained at a high P_{CO_2} by the responses of our respiratory centers. The ventilation of our alveoli could easily be more efficient than it is, but the result would be to leave the bicarbonate-carbonic acid system a much less suitable buffer for our body fluids. Since we live in an internal environment having an arbitrarily high P_{CO_2}, this permits us to have a HCO_3^- concentration high enough to provide substantial defense against H^+ and OH^-. The defenses against respiratory alkalosis are parallel to those against respiratory acidosis, involving buffering by hemoglobin and tissue-cell buffers, plus renal activity.

Defense against Metabolic Alkalosis. Suppose we inject NaOH instead of HCl. In practice we would certainly prefer some more convenient alkalinizing agent, but if NaOH were used, it would be quickly neutralized, especially by the carbonic acid that the organism is continually making in huge quantities. After the reaction, $OH^- + CO_2 \rightarrow HCO_3^-$, a slight slowing of pulmonary CO_2 excretion will serve to restore the level of carbonic acid, and the most obvious remaining aberration is the elevated bicarbonate-ion concentration. The level of this ion can be as much as doubled before an alkalosis becomes dangerous. From the mass-law equation we would predict that this doubling would halve the H^+ concentration and increase the pH to 7.7. This much our inanimate demonstration model would also accomplish, but again the living subject goes one step further. The slowed respiration continues as long as the pH is elevated, leading to an *elevated* CO_2 pressure in the alveoli and in the body fluids and a further minimization (but not elimination) of the pH rise.

Referring to Fig. 7.3, we can understand that the subject has much less further ability to withstand alkali and that a return to the normal situation requires renal activity.

Reciprocal Relationship between Serum Bicarbonate and Serum Chloride. *Acidifying action of chloride?* Reference to Fig. 7.3 also shows that as the bicarbonate concentration has increased, the chloride-ion level has decreased, and vice versa. A tendency for this reciprocal relationship to be observed in disturbances of the neutrality gives to the serum chloride determination alone, as mentioned earlier, an extremely small value (if any) in predicting the neutrality state. The possibility of other anions accumulating in acidosis (for example, ketone anions), thereby eliminating this relationship between the Cl^- and HCO_3^- levels, is usually too large to permit any conclusion from the Cl^- analysis alone.

The tendency of the serum chloride to be elevated when the bicarbonate-ion concentration is depressed has led to the uncertain concept of the *hyperchloremic acidosis,* which is satisfactory as a descriptive term but which may suggest that the chloride ion has actually *caused* the acidosis. In the hypothetic demonstration experiment of this chapter, the three conspicuous analytical end-results of the HCl injection were:

1. The halving of the bicarbonate-ion concentration
2. The increase, by an equal number of milliequivalents, of the chloride concentration
3. The doubling of the hydrogen-ion concentration (less than a doubling if continued accelerated breathing is taken into account)

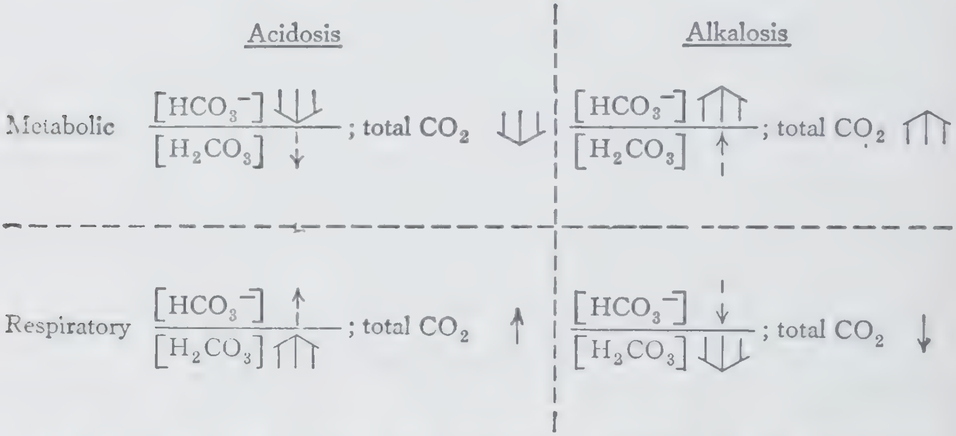

Acidosis
Alkalosis
Metabolic
$\frac{[HCO_3^-]}{[H_2CO_3]}$; total CO_2
$\frac{[HCO_3^-]}{[H_2CO_3]}$; total CO_2
Respiratory
$\frac{[HCO_3^-]}{[H_2CO_3]}$; total CO_2
$\frac{[HCO_3^-]}{[H_2CO_3]}$; total CO_2

Fig. 7.4 Showing primary and secondary changes in the bicarbonate-carbonic acid buffer system descriptive of the various types of disturbances of the acid-base balance. Note that the total CO_2 does not always fall in acidosis nor rise in alkalosis.

We can best decide whether the added *hydrogen ion* or *chloride ion* has caused these effects by testing, say, NaCl and HBr in place of HCl. Obviously the H^+ and its special properties are responsible. Probably because analyses have been made more frequently for the chloride ion than for the hydrogen ion, the causation here has not always been kept clear. The subject will be reconsidered in Chap. 10.

Does the serum CO_2 *content (or the serum* CO_2 *capacity) measure the acid-base balance?* Figure 7.4 attempts to summarize the changes in the serum bicarbonate-carbonic acid buffer system in disturbances of the neutrality.

In metabolic acidosis the entering hydrogen ion reacts with the bicarbonate ion to produce a primary decrease in its concentration (shown by the heavy arrow). Respiratory activity causes a smaller secondary decrease in the carbonic acid level, shown by the dotted arrow. Because the numerator is decreased more than the denominator, the pH must be lowered. In this instance the total CO_2 must be distinctly *decreased*. The total CO_2 content is a common and valuable laboratory determination, in which the serum is acidified and the CO_2 all brought into the gaseous form and measured.

Likewise in metabolic alkalosis, the bicarbonate ion is the primarily increased one, the carbonic acid being secondarily elevated and to a lesser degree. The total effect on the CO_2 content is an *elevation*.

Because the metabolic disturbances of the acid-base balance have attracted more attention, a mental association has been built up to the effect that a high CO_2 content means an alkalosis; a low CO_2 content, an acidosis. Note, however, the changes in the respiratory disturbances. In respiratory acidosis the retention of CO_2 and the rise in the carbonic acid are the *primary* cause. What compensatory change could minimize the pH change produced by carbonic acid accumulation? What organ could cause an elevation in the bicarbonate concentration of the extracellular fluid? Conversely, in respiratory alkalosis the net loss of carbonic acid by overbreathing is *primary*, and any tendency to compensate would decrease the bicarbonate-ion concentration secondarily.

Note that the over-all directions in the change of the CO_2 content is *downward* in both metabolic *acidosis* and respiratory *alkalosis*, and *upward* in metabolic *alkalosis* and respiratory *acidosis*. The reliability of any association between low CO_2 content and acidosis is destroyed. Some historic but mistaken conclusions have been drawn as to the character of acid-base disturbances from reliance upon the serum CO_2 content (or capacity) *alone* as an assay of the acid-base balance.

Figure 7.4 can serve as a shorthand summary of the character of the various neutrality disturbances.

How then does one determine the acid-base balance? Figure 7.5 reproduces CO_2 absorption curves applying to blood, as described in Chap. 6. These curves show how much CO_2 the blood takes up under various CO_2 pressures. The much greater uptake by whole blood than by a salt solution has been mentioned; the illustration also shows the greater uptake by reduced blood than by oxygenated blood. A pH scale has been added to the plot on the

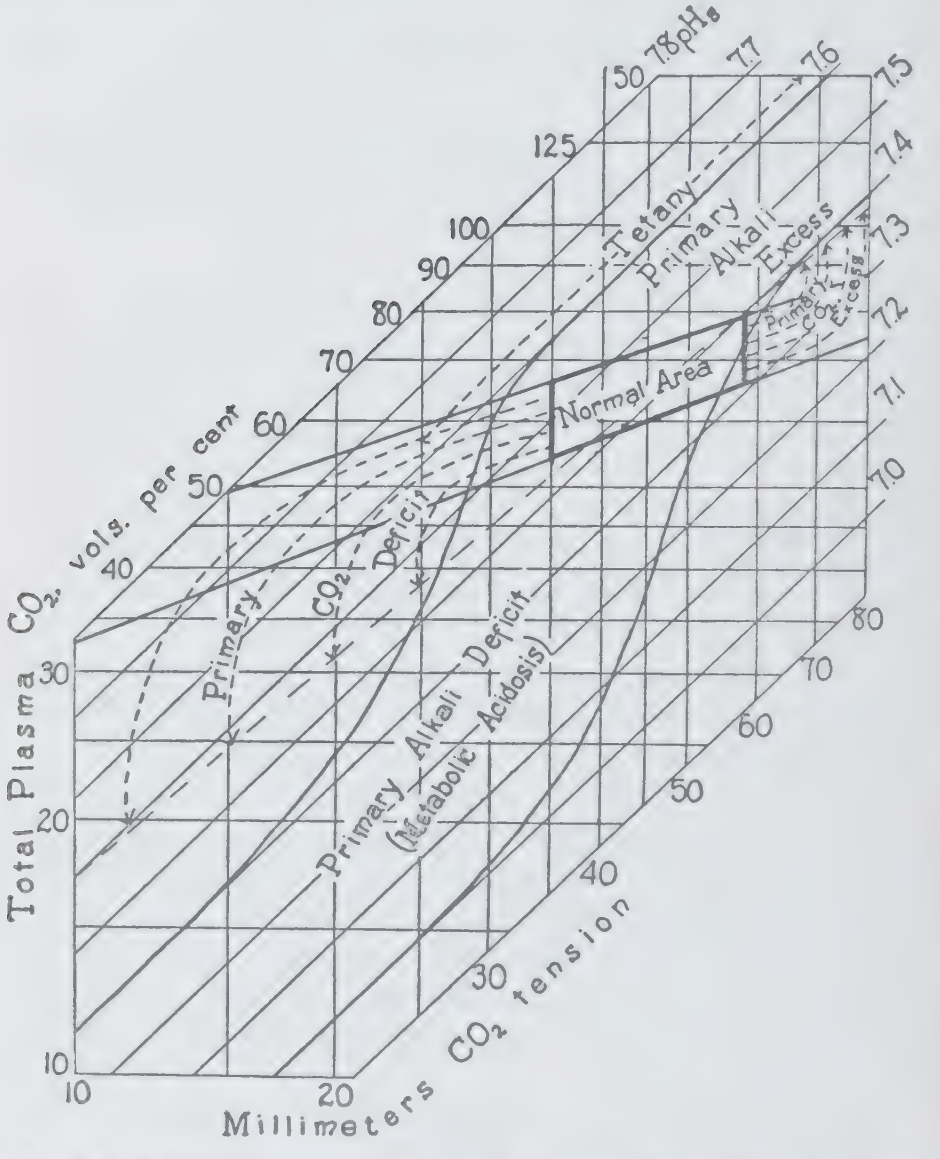


Fig. 7.5 Nature of responses to neutrality changes. (From Peters and Van Slyke, *Quantitative Clinical Chemistry,* vol. I, *Interpretations,* Baltimore, 1935, p. 944.)

following basis: The relationship between the CO_2 pressure and the CO_2 content is given by the Henderson-Hasselbalch equation as

$$\mathrm{pH} = pK' + \log \frac{[\mathrm{HCO_3}^-]}{[\mathrm{H_2CO_3}]}$$

This equation may be converted to a form dealing with more readily measured quantities; thus

$$\mathrm{pH} = 6.1 + \log \frac{[\text{total } \mathrm{CO_2} - f \cdot P_{\mathrm{CO_2}}]}{f \cdot P_{\mathrm{CO_2}}}$$

Note that $f \cdot P_{\mathrm{CO_2}}$ equals the carbonic acid concentration. This equation can be used for the serum obtained from the whole blood *after* it has been brought into equilibrium with a given CO_2 pressure, the so-called *true* serum. Whole blood analyses would include a third form of CO_2 to complicate the analysis.

Examination will show that a given ratio between the total CO_2 and $P_{\mathrm{CO_2}}$ represents a fixed pH. For example, a value of 21 for the total CO_2 and of 1 for $f \cdot P_{\mathrm{CO_2}}$ corresponds to a ratio $[\mathrm{HCO_3}^-]/[\mathrm{H_2CO_3}]$ of 20 and a pH of 7.4. Likewise a value of 10.5 for the total CO_2 and of 0.5 for $f \cdot P_{\mathrm{CO_2}}$ corresponds to the same ratio and therefore the same pH. Hence each slanting line corresponds to a definite pH as indicated.

Now we have a figure with three scales, namely, the *total* CO_2 *content*, the pH, and the CO_2 *pressure*. If we know any *two* of these for a patient's serum, we have defined the quantities considered in Fig. 7.4, and we have defined a point on this plot. By definition we have determined the patient's *acid-base balance*. Each type of disturbance of the acid-base balance occupies a given region, which we have already specified by the descriptions of Fig. 7.4. For example, in metabolic acidosis the decrease in the bicarbonate concentration is the major one, the decrease of carbonic acid the minor one. Since most of the total CO_2 is bicarbonate, this means a larger fall in the bicarbonate-ion concentration than in the CO_2 pressure. This information places us in a region of decreased pH as indicated. Similarly we can deduce areas corresponding to metabolic alkalosis, to respiratory acidosis, and to respiratory alkalosis. The courses that human subjects tend to follow in developing disturbances of a given type were carefully plotted by Van Slyke and his students and by Hastings and Shock.

Of the three analyses, the two usually selected to fix a point are the CO_2 content and the pH; determination of these two together represents a standard procedure for determining the acid-base balance.

Determinations of pH, however, have not been popular in the clinical laboratory. Although comparatively easy, considerable care in handling of blood and the maintenance of equipment is required to yield the requisite accuracy.

Several decades ago a temporary proposal was made, but subsequently withdrawn, that the necessity of making two different analyses could be avoided by the device of bringing the patient's blood to a fixed, normal CO_2 pressure, and then measuring the CO_2 content established by these conditions. This analysis is the so-called CO_2 *capacity* or CO_2 *combining power.* The procedure was supposed to cancel out the effects of respiratory disturbances because the patient's blood was brought to a normal P_{CO_2}. Therefore only metabolic disturbances presumably should be observed; acidosis by a decreased, alkalosis by an increased CO_2 capacity. At the same time the pH determination and the necessity of handling blood anaerobically were presumably avoided. (It is not an advantage, of course, that respiratory disturbances of neutrality are outside the scope of the method.)

No doubt the CO_2 accumulation resulting from a few minutes of rebreathing can be canceled out by this device, but a more prolonged respiratory acidosis leads to a compensating increase in the bicarbonate concentration which is *not* eliminated by changing the P_{CO_2}. Hence, from his blood CO_2 capacity, such a patient appears to be *alkalotic.* The reverse error appears in chronic overbreathing.

Therefore the device of bringing the blood to a normal CO_2 pressure before analyzing for CO_2 does not accomplish the purpose for which it was designed. An aberration of the procedure is to *separate the serum* of blood handled without anaerobic precautions and expose this *serum* to a normal P_{CO_2}. Such blood has lost CO_2 along a course resembling the lower of the two curved lines of Fig. 7.5. When the separated *serum* is again placed in a normal alveolar P_{CO_2}, it regains CO_2, not along this curved course but along a more nearly horizontal line describing the uptake of CO_2 by serum, leading to a lower CO_2 content and a lower pH than would have resulted from the proper procedure. This method is unsupportable. The more CO_2 lost during handling of the blood, the more serious the error will be.

Determination of the CO_2 content avoids the ambiguity of the CO_2 capacity. The necessity of including the pH determination with the CO_2 content may be set aside by other knowledge already obtained about the patient; for example, he may be known to be diabetic. To determine the *acid-base balance,* however, requires both the pH and CO_2 determinations.

LIBRARY PROBLEMS

1. Find analyses showing changes in serum bicarbonate concentration as a result of sustained elevation or depression of the CO_2 pressure. Which change is the largest percentage-wise, the change of the carbonic acid or the change of the bicarbonate concentration? Which has produced the largest percentage change in the serum CO_2? Is a serum CO_2 content of 50 millimoles per liter likely to represent respiratory acidosis?
2. Report on a paper concerning respiratory acidosis in open-chest surgery.
3. Find a textbook discussion of acid-base balance that places prime importance on the supply of "base" or "alkali" in defense against acidosis. Show how that explanation is related to the present one.
4. The proposal has been made that during acidosis acceleration of respiration stops short of returning the pH to 7.4 because this would mean that the response entirely wipes out the stimulus. Is there any valid reason why a physiological feedback correction should not eliminate entirely the aberration that set it in motion? The shape of the normal glucose tolerance curve may be considered in this connection.

8

RENAL DISPOSITION OF THE HYDROGEN ION

Nonultimate Nature of Respiratory Compensation. A glance at the serum electrolyte bar-diagram in metabolic acidosis presented in Chap. 7 told us that the lungs were unable to return the acidotic composition to the normal one. In the example given (Fig. 7.3), chloride had replaced part of the bicarbonate. To return the HCO_3^- level to normal we agreed that the extra Cl^-, relative to Na^+, had to be excreted. But Cl^- cannot be excreted alone.

Actually the kidney must excrete not only the Cl^- but also the whole dose of H^+ injected! This hydrogen ion has really not been excreted by the lungs; perhaps we may say it has only been *driven underground*. Much of it has been lost by the reaction

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

To return the HCO_3^- level to normal, we must regenerate HCO_3^- by reversing these reactions, using some of our metabolic CO_2. But in so doing, for every HCO_3^- we regenerate, we get again one hydrogen ion!

Hence the kidney has the *entire task* of excreting the HCl administered. The lungs have reduced the immediate hazard but have not made a beginning at actually eliminating the H^+ excess.

How Can the Kidneys Excrete H^+?

I. **By acidifying the urine.** The urine can be brought as low as pH 4.5 and nearly as high as pH 8. This means that the renal tubular cells can concentrate hydrogen ions to 800 times their plasma level, which is not very impressive compared with the ability of the gastric glands!

If the kidneys could produce a urine of pH 1, obviously 100 milliequivalents of free H^+ would be excreted for each liter of urine. Similarly we would have:

At pH 2.0, 10 milliequivalents H^+ per liter
At pH 3.0, 1 milliequivalent H^+ per liter
At pH 4.0, 0.1 milliequivalent H^+ per liter
At pH 4.5, 0.03 milliequivalent H^+ per liter

A common experiment is to administer to a normal person 10 g. of NH_4Cl, which contributes about 180 milliequivalents of H^+. If we had to rely upon the given rate of excretion at pH 4.5, 6000 liters of urine would need to be excreted before the resulting H^+ excess would be eliminated! Obviously very little *free* H^+ can be excreted despite the renal ability to lower the urine pH to 4.5.

Our renal ability to acidify the urine is effective only because the urine normally contains solutes that will bind H^+ as the H^+ concentration is increased from 0.00000004 to 0.000032 N. To be effective, a solute at first glance should have at least a portion of its titration curve in the interval pH 4.5 to 8. The principal solutes meeting this description are the phosphate and bicarbonate ions.

A simple-minded demonstration can be used to emphasize this point. Suppose we make a synthetic urine sample by adding some bromcresol green ($pK' = 4.7$) to a liter of water. We can add appropriate quantities of NaCl, KCl, urea, etc., if we like. Now if we try to titrate this "urine" sample with N HCl, we find from the color change that one drop of HCl already brings the pH below the permissible limit of 4.5.

Contribution of Phosphate. Now let us add to the "urine" the amount of phosphate that an adult normally excretes by this route in a day. This is about 40 millimoles, or 1.2 g. of P. If the urine pH were unmodified during its formation, roughly 80% of the phosphate would be $HPO_4^=$ and 20% $H_2PO_4^-$. Therefore let us add to the synthetic urine 32 millimoles (4.54 g.) of Na_2HPO_4 and 8 millimoles (0.96 g.) NaH_2PO_4. Now, if we try the titration again, we shall find that we can add a large amount (actually 32 ml.) of HCl before the pH passes below the limit of 4.5. Obviously the increased hydrogen-ion concentration has produced the reaction

$$HPO_4^= + H^+ \rightarrow H_2PO_4^-$$

The titration curve of phosphate is so located that practically all the phosphate can be excreted as $H_2PO_4^-$ or as much as 90% as $HPO_4^=$. This variability gives us a substantial part of our ability to adjust the elimination of H^+ to keep the body supply steady.

(The kidney certainly does not acidify urine in precisely the way we have pictured, i.e., by adding a concentrated acid HX. There is considerable evidence that the hydrogen ion competes with K^+ for excretion in the distal tubule, and further, that their competition is for Na^+ exchange. The implication is that Na^+ resorption is inextricably geared in one mechanism to H^+ or K^+ extrusion. Quite conceivably the three occupy interchangeably a single position on the same carrier, but direct evidence on this point is still lacking except perhaps in yeast (Conway). The method of *stop-flow analysis* appears to place K^+ excretion slightly distal to most of the distal tubular reabsorption of Na^+.)

The concentrative transfer of hydrogen ions into the lumen will quickly deplete their very low concentration in the plasma, but more can be synthesized by such reactions as

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

and hence as H^+ is excreted, we return the plasma HCO_3^- level toward its original value. The effectiveness of carbonic anhydrase inhibitors (like acetazolamide or *Diamox*) in preventing the acidification of the urine becomes understandable from a consideration of this reaction. If the reaction above is slowed, this source of H^+ may be inadequate. We have not, however, explained the manner in which the hydrogen ions are concentrated, which is not understood. The consequence of the increased hydrogen-ion concentration in the lumen is that the H^+ becomes bound to $HPO_4^=$ and similar buffer anions.

Bicarbonate. Another solute that varies in its state of dissociation or charge in the range of pH 4.5 to 7.8 is the bicarbonate-carbonic acid system. As usual this buffer system behaves in a fashion different than ordinary buffers.

A urine excreted at pH 7.4 may contain roughly as high a bicarbonate concentration as the plasma (Fig. 8.1). The CO_2 pressure likewise cannot be much higher than that of plasma. Now, if tubular activity brings the pH down to 6.1, one may suppose that the original total CO_2 would be divided equally between HCO_3^- and H_2CO_3. This would mean a H_2CO_3 concentration of about 15 millimolar, corresponding to a CO_2 pressure of almost 500 mm.! The urinary system is not built to maintain such a CO_2 pressure; instead the newly formed carbonic acid apparently diffuses back across the tubular cells as CO_2, and we end with small, although not constant, concentrations of carbonic acid in the urine, no matter what the pH.

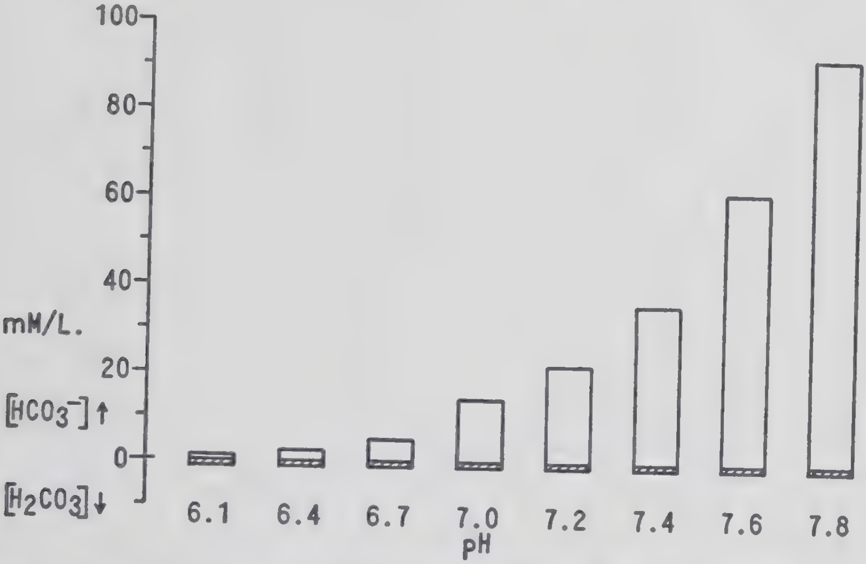


Fig. 8.1 Behavior of the bicarbonate-carbonic acid system of urine with varying pH. Upper limits are set upon the P_{CO_2}, and hence the $[H_2CO_3]$, that can be retained. The bicarbonate concentration that can be in equilibrium with this carbonic acid level is determined by the Henderson-Hasselbalch equation.

If we calculate the HCO_3^- levels corresponding to this carbonic acid level at various pH values, we see (Fig. 8.1) roughly how the bicarbonate excretion varies with the pH. Actually one can see that below pH 7.0, this solute is a minor constituent. Above pH 7.4, however, the excretion of HCO_3^- rises rapidly and becomes a powerful factor in correcting the elevated bicarbonate levels of metabolic alkalosis.

One can understand why the excretion of part of our metabolic CO_2 as bicarbonate actually combats alkalosis or hydrogen-ion deficiency from a related point of view: Every metabolic CO_2 molecule excreted as CO_2 has no net effect on the hydrogen-ion supply; but every one excreted as HCO_3^- leaves one deserted H^+ in the organism, which can no longer be eliminated by regeneration of the CO_2.

Tubular reabsorption of bicarbonate ion probably occurs not as such but in the form of CO_2, generated in the tubule by the reaction

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O$$

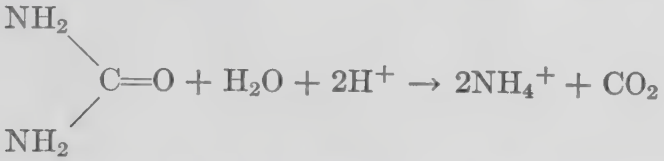
This conclusion is based upon the effective inhibition of bicarbonate reabsorption by the carbonic anhydrase inhibitor *Diamox*. A determinant influence of the amount of H^+ available on the bicarbonate reabsorption rate supports this view.

The Titratable Acidity. This is a valuable experimental technique for showing how much H^+ has been excreted by acidification of the urine. The 24-hr. urine (or in practice an aliquot portion) is titrated back to pH 7.4. The number of milliequivalents of NaOH required is taken to equal the number of milliequivalents of H^+ excreted by the acidification. If HCl instead is required to bring the pH to 7.4, the titratable acidity is *negative*, and we learn how many milliequivalents of H^+ have been *spared* by alkalinization of the urine.

The titratable acidity of a urine sample with a pH of 6, for example, is mainly accounted for by the inorganic phosphate present. If, however, the original urine has a pH of 4.5, we find more titratable acidity than the phosphate can possibly have caused. This arises from the presence in urine of undissociated organic acids (lactic, citric, hippuric, acetoacetic, etc.) whose titration curves lie partially above pH 4.5. If the urine pH is unusually low, these may be excreted partially as HA and only partially as A^-, contributing to the effectiveness of the acidification of the urine in this low region. This HA is titrated to A^- in determining the titratable acidity. Pathological increases in the excretion of organic acids will be considered in Chap. 9.

II. **Ammonium Synthesis.** Here we have an entirely different means of changing the rate of hydrogen-ion elimination to meet the variations of the H^+ excess of the organism. Many land animals excrete their waste nitrogen as urea. Some marine organisms excrete theirs as NH_3. At all pH values at which our urine can be formed, ammonia is almost entirely in the form NH_4^+, its pK being 9.4. Thus at 7.4, only 1% is NH_3; at pH 7.8 only 2.5% is NH_3 (see titration curve in Fig. 2.3). We can safely say that if we excreted all our waste nitrogen as NH_4^+ and none as urea, we would constantly be in alkalosis because NH_4^+ carries out with the nitrogen a hydrogen ion that does not accompany urea nitrogen. We also know that patients who have lost most of their ability to synthesize urinary ammonium tend to be chronically acidotic.

We can prove that an extra H^+ is excreted in NH_4^+ by writing a reaction that perhaps does not occur at all *within* the organism:



$$(NH_2)_2C{=}O + H_2O + 2H^+ \rightarrow 2NH_4^+ + CO_2$$

This reaction proves that each ammonium ion *contains a* H^+ *not present in urea,* but the actual reactions primarily concerned in ammonium excretion are shown in Fig. 8.2.

Glutamine is the most abundant free amino acid of plasma and most tissues. The other product of the cleavage besides NH_4^+ is glutamate, which also occupies a central place in amino-acid metabolism.

Man may excrete almost no ammonium ion, or he may excrete a very substantial part of his nitrogen in this form, thereby accounting for a considerable excretion of H^+. This adjustment shows a capacity for handling H^+ in acidosis greater than the process of urinary acidification. Note that this is a separate mode of eliminating H^+, which in contrast to urine acidification is essentially as effective if the pH is 7.4 or 5.4. True, a low plasma pH ordinarily leads to both acidification of the urine and ammonium synthesis, so that a subject is likely (but not certain) to show both a high titratable acidity and a high urinary NH_4^+ at the same time. The ammonium excretion rate correlates more closely with the urine pH than with the plasma pH when the two are dissociated. This situation has led to suggestions that ammonia distribution is controlled by the small percentage in the permeating NH_3 form. This would be much less at pH 4.4, for example, than at pH 7.4. If NH_3 is equally distributed between two phases separated by a membrane impermeable to NH_4^+ (Fig. 8.3), the more acid solution would have a higher ($NH_3 + NH_4^+$) concentration. The renal production rate for NH_4^+ must, however, also be considered as a factor in NH_4^+ excretion.

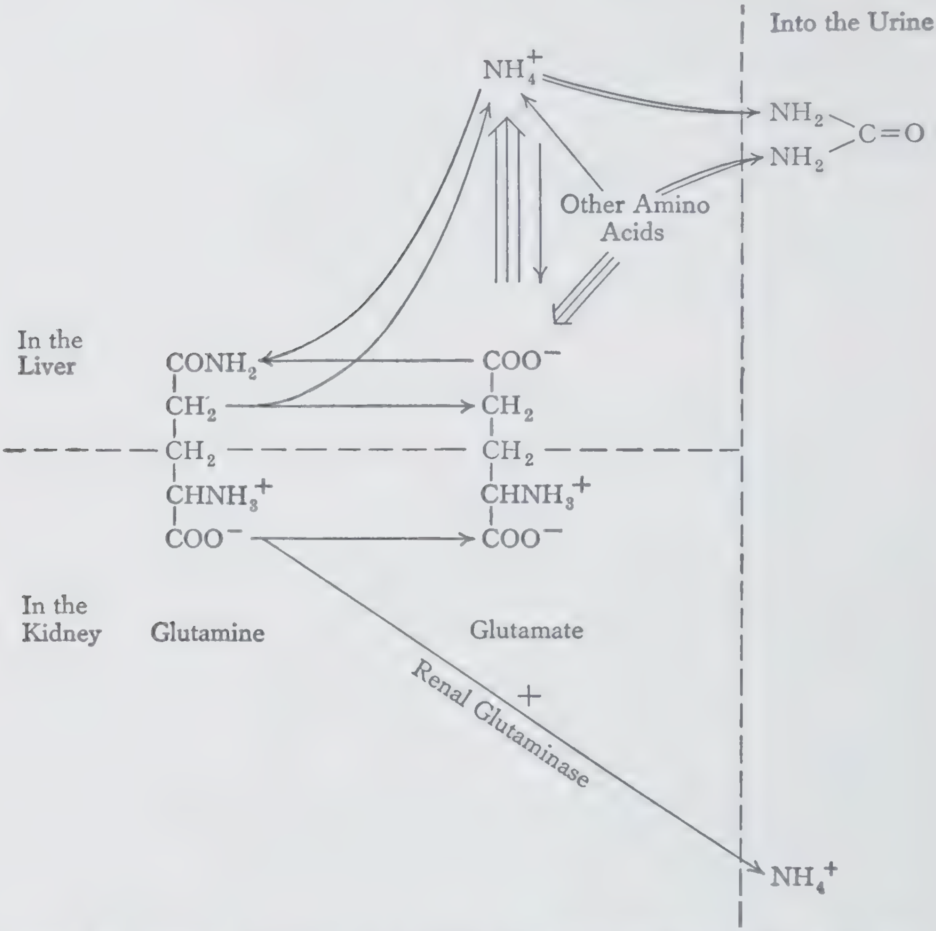


Fig. 8.2 Urinary ammonia is formed at the expense of urea. The withdrawal of plasma glutamine through glutaminase action increases the net glutamine formation in the liver and thereby decreases urea formation.

If this theory is correct, NH_4^+ excretion is driven by the H^+ pump, and all modes of H^+ excretion are in some degree reunified. Although they are likely to occur together and both may require the separate transfer of H^+ into the tubular urine (if NH_4^+

actually passes through as NH_3), the different chemical basis of these two modes of H^+ elimination must nevertheless be understood. This difference is emphasized by the failure of the titratable acidity to include the H^+ excreted as NH_4^+. Instead we must add the 24-hr. *titratable acidity* to the 24-hr. *ammonium excretion* to calculate the *total urinary* H^+ *excretion* for the day. The two mechanisms may be lost independently in renal disease, ammonium synthesis being the more frequently lost.

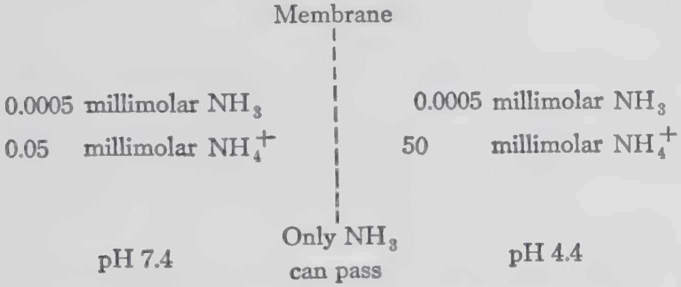


Fig. 8.3 Hypothetical example to show how NH_4^+ will be distributed asymmetrically between two phases of different pH, if only NH_3 can pass through the membrane. If the *pK* is 9.4, at pH 7.4, $NH_3/NH_4^+ = 1{:}100$; at pH 4.4, $NH_3/NH_4^+ = 1{:}100{,}000$. The NH_3 concentration is the same on the two sides, but the concentration of NH_4^+ is determined by the Henderson-Hasselbalch equation. Thus the H^+ pump would actually concentrate NH_4^+.

The efficient operation of these renal adjustments of the neutrality requires a certain level of renal blood flow. In dehydration the decreased plasma volume interferes with or interrupts fine adjustments, and neutrality maintenance frequently fails. Why the result is usually acidosis will be considered in Chap. 9.

Another renal limitation in neutrality control is seen in the *paradoxical aciduria* that may be observed in the dehydrated alkalotic patient. The normal response to extracellular alkalosis will cause an alkalinization of the urine. One may rationalize that the stimulus toward conservation of alkali-metal cations arising from their deficiency predominates over the contradictory stimulus toward hydrogen-ion conservation, or one may instead merely conclude that the dehydration imposes a limitation here also on the correction of the prevailing trend.

LIBRARY PROBLEMS

1. Note that it is the *normal, daily* urinary phosphate excretion that contributes so largely to the efficacy of the acidification of the urine in eliminating extra quantities of H+. If the normal daily allowance of P is 1.5 g., but the normal adult excretes only 1.2 g., does this imply he is getting less than the recommended amount? An acidosis will cause some bone dissolution and therefore a further increase in phosphate excretion, which enhances the effectiveness of a given degree of urine acidification. Do you think this *extra* H+ excretion should be considered to be *net, ultimate elimination of hydrogen ion?*
2. From the foregoing discussion, one may be tempted to say that the urinary phosphate provides more protection against acidosis than against alkalosis. But suppose that a given population, on their usual diet, has an average urine pH of 6.5. Would the presence of phosphate in the urine give them more defense against unusual acidifying or alkalinizing influences? Against which hazard is the urinary bicarbonate-carbonic acid system more effective?
3. In determining the urinary titratable acidity, the usual technique is first to dilute 25 ml. of urine to 200 ml. Otherwise $Ca_3(PO_4)_2$ precipitation is likely to occur. What error will this precipitation cause? In the Folin method one titrates to the phenolphthalein endpoint at a pH of perhaps 8.5. Comment on the relative significance of the latter titration.
4. Urine is by no means constant in its salt concentration or *ionic strength.* The pK_2 of phosphoric acid changes rather greatly with the ionic strength. Taking this into account, under what conditions would the titratable acidity need to be performed to obtain a precise measure of the hydrogen ions added in urine formation? The problem can be illustrated by calculating the milliequivalents of NaOH required to bring, say, 30 millimoles of $H_2PO_4^-$ to a pH of 7.4 if the pK_2' is (a) 6.7, (b) 6.9.
5. Examine textbook schemes that are intended to show how hydrogen ion is excreted by the kidney. Does the scheme suggest how H+ is concentrated or how it is exchanged for Na+ or K+? Does it provide a source of the H+?
6. A recent report proposes that uric acid stones are a consequence of a defect in renal ammonium synthesis and not in purine metabolism. A chronically low urine pH is considered to lead to precipitation. Prien has shown how the solubility of uric acid changes with pH. Discuss this proposal carefully.
7. Do ammonium synthesis and urine acidification always contribute in a fixed proportion to hydrogen-ion elimination, or do their proportional contributions vary according to the way in which acidosis has been produced?

8. Jorgensen has proposed a single titration to measure the H+ excreted into the urine. To 10 ml. of urine is added 1 ml. of *N* HCl; the urine is boiled, formaldehyde is added, and the urine then cooled and titrated to pH 7.4. The excreted H+ is taken to equal the excess of NaOH required over a parallel experiment, using water instead of urine. Considering the usual forms in which H+ is excreted, explain how this can be claimed to measure net H+ excretion. Could any normal urinary constituents that really do not represent net H+ excretion be included in the titration?

9

ENDOGENOUS PRODUCTION AND CONSUMPTION OF HYDROGEN IONS

The production of acidosis by administered HCl and of alkalosis by administered NaOH or $NaHCO_3$ are rather obvious consequences. The same may be said for the effects of losses of gastric HCl by vomiting or of alkaline secretions in diarrhea. A classical study by Gamble, Ross, and McIver in 1925 showed how the effect of ligation of the alimentary tract changed from an *alkalinizing* one, with ligation at the pylorus, to *acidifying,* with ligation at lower levels. Whether the vomiting of gastric juice really is alkalinizing depends upon whether the vomited juice actually is acid, which is not necessarily the case. The effects in general are those that would be expected from the nature of the material lost or gained.

Much less uniformly understood are the production and consumption of hydrogen ions by reactions occurring *within the organism.* The origin of diabetic and renal acidosis, as well as the influences of such agents as NH_4Cl and sodium lactate on the neutrality, involve the ideas to be discussed here.

Very large quantities of H^+ are *produced* in metabolism and very large quantities are *consumed.* From these facts the pessimistic conclusion has been occasionally drawn that a *balance* of *intake* and *output* of hydrogen ion cannot be constructed, or even that such a balance is irrelevant to neutrality control!

Let us examine how endogenous H^+ production occurs. One of the two following reactions is already familiar to us:

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^- \qquad (9.1a)$$

$$\text{Glucose} \rightarrow 2\,\text{lactate} + 2H^+ \qquad (9.2a)$$

Both reactions release hydrogen ion. In reaction 9.2a you may prefer to consider lactic acid as an intermediate, which is acceptable as long as we remember that the lactic acid as such cannot remain as a tissue constituent because of its low *pK*.

These two reactions are alike in a second respect: Their release of H^+ is *transient*. If we complete the metabolism of each intermediate, we see that the hydrogen ions formed are again eliminated; thus

$$H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow H_2O + CO_2\uparrow \qquad (9.1b)$$

$$\text{Lactate}^- + H^+ + 3O_2 \rightarrow 3H_2O + 3CO_2\uparrow \qquad (9.2b)$$

Many other examples of the same kind may be found, for instance:

$$CH_3(CH_2)_{14}COO^- \rightarrow 8CH_3COO^- + 7H^+ \qquad (9.3a)$$

$$CH_3COO^- + H^+ + 2O_2 \rightarrow 2H_2O + 2CO_2 \qquad (9.3b)$$

The principle illustrated here is a chemical one: When a neutral organic compound in an aqueous system is converted to an anion, a hydrogen ion will also be formed unless some other cation is a product. This applies in the test tube or in the organism; when the anion is again converted to neutral products, the hydrogen ion will be consumed. The poor biochemical habit of writing equations that show substances in states of dissociation that can scarcely exist at body pH often obscures these relationships.

Metabolism therefore does not usually lead to a *net* change in hydrogen-ion supplies. With certain exceptions to be considered in subsequent paragraphs, only as the rates of various reactions are changed disproportionately, so that intermediates *accumulate* or are depleted, is there an effect on neutrality.

Net H^+ Production or Consumption. If, however, we can isolate the *anion-producing reaction* from the *anion-destroying reaction*, or vice versa, we can obtain net H^+ production or consumption. In insulin deficiency, for example, the production of certain anions runs well ahead of their consumption (again omitting intermediate stages); thus

$$CH_3(CH_2)_{14}COO^- + 7O_2 \rightarrow 4CH_3COCH_2COO^- + 3H^+ + 4H_2O$$

The ketone anions accumulate, and therefore hydrogen ions must also accumulate (not necessarily in free form), but these will be eliminated if and when acetoacetate oxidation again catches up with its production. Part of the ketone anions will be lost into the urine, however, leaving *deserted* hydrogen ions that cannot be removed catabolically even if enough insulin is given subsequently to permit catabolism of accumulated ketone acids. Note that the acidifying effect of the loss of HCO_3^- into the urine is highly analogous; here again, hydrogen ions are deserted so that reaction 9.1b cannot destroy them.

In contrast we isolate the hydrogen-ion-destroying reaction when we *combat* acidosis by the administration of sodium lactate. In this case we secure only the *second* stage of the process, i.e.,

$$CH_3CHOHCOO^- + H^+ + 3O_2 \rightarrow 3CO_2 + 3H_2O$$

When lactate is oxidized, a hydrogen ion must inevitably be withdrawn from the environment, accounting for the alkalinizing action. Sodium lactate in one-sixth molar solution is one of the most convenient therapeutic intravenous alkalinizing agents; it has the advantage over $NaHCO_3$ in that it is stable enough for heat sterilization.

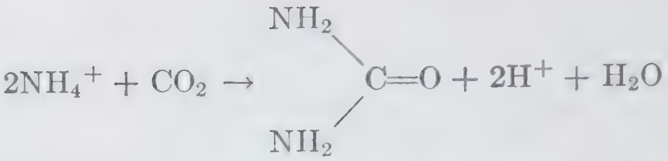
If we consume food acids like lactic or citric acid, there is *no net effect* upon the neutrality because the hydrogen ion is eliminated when the organic anion is catabolized. Incombustible acids like tartaric or benzoic acids will, of course, be acidifying like HCl. Citrus fruit juices contain not merely citric acid but also some potassium citrate; when this citrate ion is oxidized, as for the lactate ion, an alkalinizing effect will result. Most vegetable foods have an alkalinizing action through the presence of combustible anions.

Accumulation of Organic Anions in the Blood. A number of organic anions, such as lactate, pyruvate, acetoacetate, and citrate, are normally present in the serum to a total of about 5 or 6 milliequivalents per liter and presumably permit metabolic exchange among the cells of the organism. Intense muscular exercise may increase the lactate concentration very rapidly; the bicarbonate concentration falls correspondingly as though lactic acid had been added to the blood. When acetoacetate and β-hydroxybutyrate are being produced more rapidly than they can be consumed, their concentration rises more gradually in the plasma, but in this case the rise is quite as much at the expense of chloride as of bicarbonate. In either case the diminution of the bicarbonate level represents a temporary handicap to the respiratory neutrality control. One should remember that the influence of these organic anion accumulations upon the neutrality will be reversed when they are subsequently catabolized.

Acidifying Effect of Protein Foods. Another instance where we isolate a H^+-producing reaction is in the oxidation of "neutral" sulfur; for example, sulfur as it is present in cystine, cysteine, or methionine. Here substances of no net charge are oxidized to neutral excretion products *plus sulfate ion.* Two hydrogen ions are inevitably formed along with the sulfate ion. Obviously the organism cannot excrete sulfate in the form of gaseous SO_3, in analogy to the excretion of HCO_3^- as CO_2; nor as H_2SO_4. These hydrogen ions are deserted in the body and impose a burden upon the special facilities for excreting hydrogen ion discussed in Chap. 8. Some writers have proposed a similar role for the phosphorus of protein foods, but this is a different situation, since phosphate in food is usually not found in a nonanionic state.

The normal food intake of people over much of the world contains enough sulfur-containing protein to cause a net metabolic production of acid (as shown by an average urine pH below 7.4). Furthermore, under conditions of partial or complete fasting (for example, in disease), the fuel of the body is a high-protein one because our own tissues are being consumed. Accordingly the usual strain upon our neutrality is on the *acid* side. Therefore, in dehydration, where the kidney is handicapped in its excretion of excess H^+, the trend is almost always an acidotic one. The same situation usually applies in the acidosis of renal disease. "Hyperchloremic" acidosis frequently has this origin.

Acidifying Effect of NH_4Cl. Endogenous net production of H^+ also occurs when NH_4Cl is fed. The liver clears the NH_4^+ arriving in the portal blood by an over-all reaction that releases one hydrogen ion for each NH_4^+ utilized, thus



$$2NH_4^+ + CO_2 \rightarrow (NH_2)_2C{=}O + 2H^+ + H_2O$$

(Note that we do not need to trace these reactions through the detailed pathways actually followed in metabolism. A balanced equation leading from the reactants to the products will establish whether H^+ is formed or consumed.)

NH_4Cl accordingly serves as convenient acidifying agent. The acidifying effect of the conversion of NH_4^+ to urea is a necessary converse to the alkalinizing effect of substituting NH_4^+ excretion for urea excretion, as discussed in Chap. 8.

Calcium chloride by mouth also has an acidifying action because the calcium enters into reactions in the intestine. These reactions are of the following character:

$$3Ca^{++} + 2HPO_4^{=} \rightarrow Ca_3(PO_4)_2 + 2H^+$$

Obviously a therapeutic correction of the neutrality by agents like sodium lactate or NH_4Cl can make unnecessary the renal correction discussed in Chap. 8. Fortunately a completely exogenous correction is rarely necessary; this probably can be achieved only by successive approximations because of the uncertainties about the distribution of the hydrogen ion, uncertainties that cannot be dispelled by blood analysis.

LIBRARY PROBLEMS

1. Consider the metabolic fate of administered benzoic acid. Should this compound have an acidifying effect? Should sodium benzoate feeding have such an effect? Would fed aspartic acid have a net acidifying effect? What form of glutamic acid is sometimes administered to secure a net acidifying effect?
2. β-Hydroxybutyric acid has a pK' of 4.4. This means that in a urine at pH 4.5, nearly one-half of it is excreted in the undissociated form HA; and the remainder as the dissociated anion A^-. Does the part excreted as HA have an effect on the neutrality different from the part that is oxidized to CO_2 and H_2O in the body? Which is included in the titratable acidity, the quantity of β-hydroxybutyric acid or of dissociated β-hydroxybutyrate? Does the increase of the

titratable acidity resulting from the presence of this substance in ketosis mean that the body is getting rid of more hydrogen ion than usual, or merely that *renal elimination* of H+ has been substituted for *catabolic consumption* of H+?

3. The life-raft survival studies of Gamble and Butler sought to determine the best distribution of the limited storage space available in a life raft between water and foods. They concluded that about 500 ml. represented approximately the minimum water requirement but that body water would be actually better conserved if the next 100 ml. of space were given over to sugar or a sugar food instead of water. Why should this be the case?
4. Cooke recently classified laboratory analysis contributing to the management of disorders of body water and electrolyte as follows:

   a. Measurements essential to deficit therapy
   b. Measurements helpful in deficit therapy
   c. Measurements essential to maintenance therapy

   Indicate which analysis he placed in each group and why.
5. It has been suggested that fruit and vegetable foods are high in potassium relative to sodium because they are *cellular* in composition. Investigate this view. Is sodium an essential element in plant nutrition?
6. Describe one or more methods for determining ketone bodies. Is the analysis frequently made on blood serum? Does the method respond to all three of the substances usually included in the term *ketone bodies?* What is the proprietary test known as "Acetest"?

10

ANION-CATION BALANCE

The account of neutrality control in Chap. 9 summarizes our common heritage of experiment and observation, but the variations of terminology in this field are so large that the student can find authoritative summaries of the same subject in various textbooks that read entirely differently. He will note especially a much greater emphasis upon other inorganic ions than has been so far included here. This chapter seeks to add an aspect that the student needs to understand, and it discusses the basis on which this aspect sometimes receives much more emphasis than we have given it.

Why Are Anions Called Acids and Cations Bases? From the chemical and alchemical past comes one basis for this terminology. An alkali metal or its oxide reacts with water to form an alkaline solution; a nonmetal oxide forms an acidic solution. Once the sodium ion and the sulfate ion are formed, however, they have *none* of this property left, and certainly the properties of NaOH or H_2SO_4 cannot be imputed correctly to these ions.

A more rational basis for the terminology, however, may be found. Biological fluids contain two kinds of anions, *buffer* anions and *nonbuffer* anions. The latter are frequently called *fixed anions*, i.e., anions whose state of charge is fixed over the relevant range of pH. In contrast the cations are essentially all *fixed cations*. (Free histidine$^+$ is an example of a buffer cation, but such cations are not particularly abundant compared with Na^+, K^+, etc.)

When a strong acid H^+X^- is added to a biological solution of the composition described, the H^+ reacts with the buffer anion A^-

$$H^+ + A^- \rightarrow HA$$

If the HA is H_2CO_3 it is promptly excreted. One of the principal observable consequences upon analysis of the solution is the increased level of fixed anion, X^- now having replaced the buffer anion in the bar diagram (Fig. 10.1).

Hence we come to recognize as acid a biological solution that has a fixed *anion* level out of the usual proportion to the *cation* level. When the kidney corrects this situation, the fixed anion concentration of the *plasma* is again lowered to the usual value. In the meantime the level of fixed anion of the *urine* has been increased, and its level of buffer anion has been lowered by reaction with the excreted H^+. Thus the urine has also gained in proportionate fixed-anion content. Therefore it appears that the *acidity of the plasma has been assumed by the urine by the transfer of fixed anions to it.* We have seen in the preceding reaction that *the hydrogen ion* has also been transferred, but this is perhaps less obvious because it passes at an enormous flux through a very small concentration of its free form.

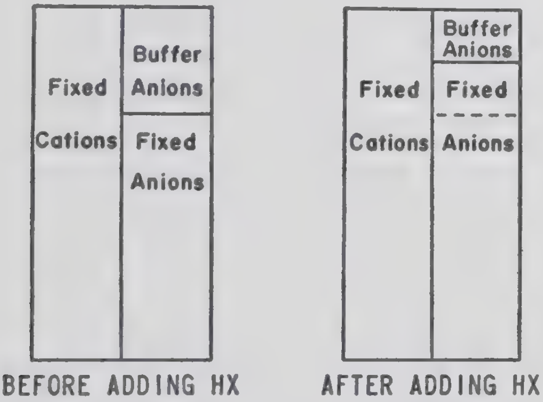


Fig. 10.1 Illustrating the effect of strong acid addition to a typical biological fluid. The most obvious change is the decreased buffer anion concentration and the replacement of this buffer anion by the fixed anion of the added acid.

Conversely, a biological solution with a proportionately increased *cation* concentration is recognized as *alkaline;* the correction of this alkaline state is marked by *a transfer of the cation excess* to the urine.

From these examples we can understand that the relationship between the fixed-cation and fixed-anion levels is entirely usable as a convenient indicator of neutrality, especially for any biological solution as well known as our blood serum. Because pH determinations have not been made as regularly as electrolyte analyses, much more familiarity has been gained with this approach. Going only a step further, one may come to think of the cations as *bases* and the fixed anions as *acids*, but this is a long and dangerous step that begins to attribute *acidifying properties* to the chloride ion and *basic properties* to the sodium ion. A medical scientist would never think to attribute the buffering by a phosphate buffer, which he makes and uses in his laboratory, to the sodium ion that it contains; but under the guidance of tradition in his teaching, he may ascribe *in vivo* neutralizing powers to the sodium ion.

The only reason the neutrality can be described by the relationship between the fixed anion and the cation levels is that this relationship can give us *indirectly* the concentration of the *buffer anions*. These, together with the H^+ which they tend to bind, are the real actors in the drama. Certainly no one would seriously propose discussing neutrality regulation in terms of the hydrogen ion alone without reference to the buffer anions. As long as we treat the fixed-ion levels as a *reflection* of the hydrogen ion distribution rather than the *cause* of it, we have gained a valuable ancillary approach.

Parenthetical Comment on the Ionic Theory. One source of confusion, probably now becoming rare, is a failure to separate clearly the concepts of covalence and electrovalence. In a solution of sodium bicarbonate, the sodium ion is entirely dissociated from the bicarbonate ion, whereas the hydrogen ion is a firmly bound part of the bicarbonate ion, which has little tendency to dissociate unless the H^+ concentration is very low. In short, the relationship of the Na^+ to the carbonate radical is very different from that of the H^+. The same is true in hemoglobin at pH 7.4. At this pH several hydrogen ions have dissociated from the globin to leave it an anion; there are a good many more firmly bound hydrogen ions still on the protein molecule. The hemoglobin anion is present *as such, free* and *not bound to the potassium ions* also in the solution. For example, the hemoglobin moves electrophoretically in an electric field as an anion, without the potassium ion. Hence any equations of this character

$$HHbO_2 + KHCO_3 \rightarrow KHbO_2 + H_2CO_3$$

actually appear to reflect a reluctance in accepting the Arrhenius theory and in dealing with the particles really concerned. One does not need to fear that electroneutrality will be lost by neglecting the cation as long as only electrically balanced equations are postulated. In the past the inclusion of the cation has been thought to *simplify* the subject; for the present-day student, trained in chemical bonding and ionic theory, it only complicates it.

Are Na^+, K^+ and Cl^- Really Inert? A substance that is acidifying to a water solution must react to release hydrogen ions. No such reaction for Cl^- in water is known. A substance that is alkalinizing must cause the binding of hydrogen ions. It is equally hard to imagine Na^+ and K^+ doing this. Important biological reactions of cation transport may be discovered in which these cations, in combining with their carriers, have the reverse effect of *displacing* H^+ to produce *acidifying* effects. Even such interactions, however, are probably highly transient.

The Buffer Anions Are Not Inert. They Are Bases. Responsibility for the alkalinizing action of such an agent as $NaHCO_3$ must be borne either by the sodium ion or by the bicarbonate ion. Lack of understanding of the properties of the bicarbonate ion in the past has perhaps misdirected attention to the sodium ion. The modern student presents a pre-medical training in chemistry that permits him readily to grasp that the bicarbonate ion (and similar buffer anions) are really basic in that they tend to remove free H^+ from solution; thus

$$HCO_3^- + H^+ \rightarrow H_2CO_3$$

Furthermore, nothing he has learned about the chemistry of the sodium ion permits him to understand how it can be alkalinizing.

The Parallel Explanations of Neutrality Phenomena. Under the convention outlined in the preceding section one may say that vomiting of gastric juice tends to cause alkalosis because of the excess chloride loss and that loss of intestinal secretions is acidifying because excessive proportions of Na^+ and K^+ are lost. One may say that a solution of "physiological" saline placed in the high P_{CO_2} of the physiological environment is acid because it has an excessive (relative) chloride content. To permit it to become neutral, a gap is needed between the Na^+ and Cl^- concentrations which may be filled with bicarbonate ion. (This temporarily overlooks the fact that forming HCO_3^- from H_2CO_3 requires the *removal of* H^+, so that we must remove H^+ *and* Cl^-, not just Cl^- from physiological saline to make of it a solution neutral in the physiological environment.)

We may say that the kidneys correct systemic acidosis by excreting excess fixed anions. As the urine is acidified, the $HPO_4^=$ ion is changed to the singly charged $H_2PO_4^-$ ion, which cuts in half the number of milliequivalents of anion excreted as phosphate, making room for more fixed anions to be excreted, or permitting the *conservation of fixed cations*. An increased titratable acidity is apt to be interpreted in terms of the cations spared rather than the hydrogen ion excreted. (This view temporarily overlooks that one hydrogen ion is eliminated for every equivalent of fixed anion excreted or fixed cation conserved and that the value of the ion analyses is to reflect the H^+ excretion.)

Likewise we can say that ammonia synthesis combats acidosis by substituting the synthetic NH_4^+ cation for alkali-metal cations (again neglecting the increased hydrogen-ion excretion). We may say that $Na^+HCO_3^-$ excretion combats alkalosis because the alkalinizing Na^+ is eliminated, that sodium lactate administration is alkalinizing because fixed cation is being supplied without fixed anion, that dietary $CaCl_2$ is acidifying because the anion is absorbed more completely than the cation, that NH_4Cl is acidifying because only the anion is contributed to the system.

One may also say that ketosis is acidifying because fixed anions (at least invariant in charge in the range of the plasma pH) are being accumulated and because in their excretion they drag fixed cations from the body (ignoring the deserted hydrogen ions they leave behind).

Limitations of the Cation-Anion Viewpoint. If we limit ourselves to this point of view, we shall be at a loss to interpret the *metabolic production* and *consumption* of hydrogen ions when there is no net change in the fixed ions of the body. In this area these special tools of fixed-ion analysis are inadequate. Furthermore one is led to regard a failure in excreting a metabolic product completely (for example, sulfate or phosphate) as a fixed anion accumulation and therefore acidifying. Now the *production* of sulfate is obviously acidifying, but one cannot readily imagine why sulfate should be any more acidifying if not quite all is excreted. If there is a renal difficulty in excreting chloride, or fixed anions in general, but a normal ability to excrete H^+, one would anticipate that as $HPO_4^=$ is converted to $H_2PO_4^-$ in the tubules, the incidental adjustment in B^+ and Cl^- excretion would involve *more* B^+ conservation and *less* Cl^- output than usual. Edema could result, but any failure to correct acidosis would more plausibly be attributed to actual difficulty in excreting hydrogen ions rather than fixed anions. For any acid (for example, a ketone acid or sulfuric acid) to displace bicarbonate ion, it must supply hydrogen ion to convert the bicarbonate to CO_2 and H_2O.

Retention of Flexibility as to Viewpoint. Although the writer is attempting to support one point of view more than the other, the present-day student cannot consider himself trained in the subject until he can communicate at will in either terminology without confusion and yet avoid the fallacy of actually regarding anions as acidifying and cations as alkalinizing. Otherwise he will be handicapped both in communication and in reasoning from inorganic ion analyses; for example, by the approach proposed in Fig. 3.3.

LIBRARY PROBLEMS

1. Find a classical paper proposing the conclusion that in the so-called alkalosis of potassium deficiency the body considered as a whole is actually acidotic. The changes in the results of three urinary analyses during convalescence from K^+ deficiency served to prove this. Show that each of these changes actually measures an increased net loss of H^+ from the experimental animal.
2. Find and discuss a paper in which evidence is reported that hydrogen

ions introduced into an experimental animal are effectively present in the animal (even though largely combined) until they are excreted, i.e., that a balance between hydrogen intake and output can be constructed.

3. Find and report a discussion by two medical students in the journal *Surgery, Gynecology, and Obstetrics* discussing the relative acceptability of the unitarian and dualistic approaches to neutrality regulation.
4. Find and discuss a 1954 editorial tracing the historic origin of the use of the terms *acid* for anion and *base* for cation.
5. One is advised to analyze juices aspirated in large volumes from the alimentary tract (for example, post-operatively) for Na^+ and Cl^- and to repair the losses of each; for example, by sodium chloride or sodium lactate solutions. Will this procedure tend to correct the neutrality-disturbing effects of such losses? Show why.
6. Find a paper presenting the concept of the *blood buffer base*. What narrowness of the concept of the so-called *alkali reserve* does this proposal correct? Does this concept omit or minimize the influence of the hydrogen ion in neutrality control? Does the term refer to buffering cations? What alternative terms could serve for the *blood buffer base?*
7. The dehydration of diabetic acidosis has been attributed to the withdrawal of extracellular Na^+ because of the excretion of the ketone acids, mainly as their alkali-metal salts. Find and diagram the approximate serum electrolyte framework of a patient in severe diabetic acidosis. Which has been more seriously depleted, the serum Na^+ or the serum Cl^-? Does this support the view that extracellular electrolyte has been depleted primarily by withdrawal of sodium acetoacetate? Consider also that a good deal of the extracellular Na^+ has been lost into the cells.

11

SMALL NITROGEN COMPOUNDS—DISTRIBUTION OF AMINO ACIDS

The small-moleculed nitrogen compounds of our extracellular fluid have been grouped together under the term *the nonprotein nitrogen,* or NPN. From this association these substances have gained a crude collective significance, namely, as endproducts of nitrogen metabolism, which actually is contributed mainly by a few members of the group. This description is highly inapplicable to most of the others. Instead, like the amino acids, these may be coins of exchange among the cells.

The more abundant NPN compounds in the plasma, in order, are: urea, amino acids (collectively), uric acid, and creatinine. No one knows where such a list would end if we were to include all the nitrogen compounds invariably present. Among the compounds that will certainly be present in the circulating fluid are the essential *nutrients* and *hormones* which must reach the cells by this route. In addition, compounds that are synthesized or destroyed much faster in certain tissues than in others will be present in the plasma, so that specialization of function occurs, as illustrated by the amino acids.

The amino acids enter the body through the intestinal wall and leave it mainly by net deamination (leading to urea formation) in the liver. Portions must reach all cells. Furthermore the liver interconverts amino acids more rapidly than certain other tissues, which means that these latter tissues may well receive their whole requirement of amino acids (both essential and nonessential) from the circulation, rather than make their own nonessential ones.

In the final analysis, however, the circulating fluid will contain all metabolic intermediates that are permitted to escape from the cells, whether transfer from cell to cell *is* or *is not* necessary. Suppose, for example, that every cell in the body could form its own pyrimidines. These compounds would then not need to circulate. This does not, however, necessarily establish that they *would not* circulate.

Furthermore we can judge neither the *rate* nor the *necessity* of exchange of a substance among cells from its level in the extracellular fluid. In the plasma the protein nitrogen level is usually over 1000 mg. per 100 ml.; the urea N, perhaps 15 mg. per 100 ml.; and the fasting α-amino acid N level less than 5 mg. per 100 ml.; yet there can be no question that the latter collectively represent the *fastest* and the former the *slowest* nitrogen stream of the three. The urea N arises largely from amino acid N, so that the traffic of the latter to the liver must be at least as fast as the movement of the former from the liver; but the amino acids also move from the intestinal wall to the cells, and from one cell to another to be incorporated and reincorporated into proteins, in addition to moving to the liver to be destroyed. The urea, in contrast, is obligated only to a one-way journey to the kidney; although it enters various cells, it is not trapped by them.

The abundant plasma proteins also have long been suspected of serving as coins of nitrogen exchange among tissues, but their significance lies largely elsewhere; as discussed in Chap. 13, they are only relatively sluggish precursors of tissue protein, and that probably by way of the free amino acids.

One way of looking at the matter is to conclude that a substance is present at a high level in the extracellular fluids when the cells have limited capacities to deplete it.

Amino Acid Absorption. We can illustrate *normal* digestion of proteins by the contrast with *defective* digestion. Figure 11.1 illustrates the much smaller rise of the plasma glycine after a gelatin test meal in a child with pancreatic fibrosis than that in a control patient. The upper curve shows the effect of giving the patient coated pancreatin granules along with the gelatin test meal. The added enzyme accelerated the hydrolysis of the protein so that glycine entered the circulation much more rapidly than it was withdrawn from it. The pepsin and intestinal peptidases of the child with fibrocystic disease eventually digested most of the protein fed; the difference we see here is a consequence of a *slower rate* of protein hydrolysis resulting from the absence of the pancreatic proteases (Shwachman).

If, instead, the difficulty were a malabsorption syndrome, even feeding glycine in the *free* form would result in only a small rise of the plasma glycine level.

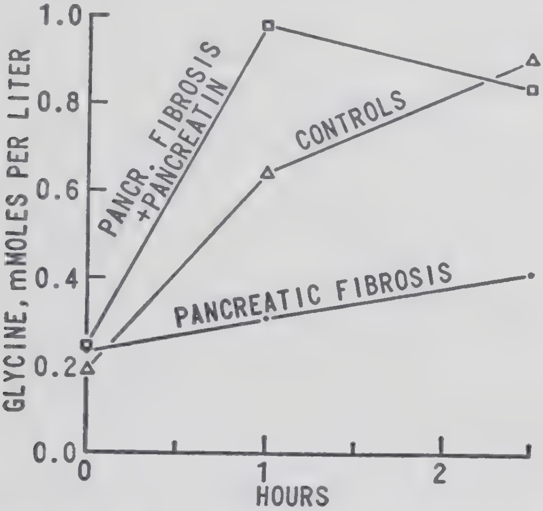
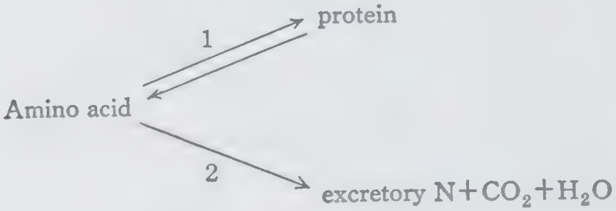


Fig. 11.1 Plasma glycine levels after feeding a gelatin test meal. In the absence of the pancreatic enzymes, the slower digestion, and consequent slower entrance of glycine into the blood, leads to a much smaller rise in the peripheral plasma glycine level. If "enteric-coated" pancreatin granules are included with the test meal, the rise in plasma glycine is increased to normal.

The normal intestinal wall *concentrates* amino acids of the L-family from the mucosal to the serosal side, transferring them against a concentration gradient. The various amino acids compete with each other for absorption, introducing complexities that are not yet fully interpreted (Wiseman). Even when analysis shows a peptide-rich solution in the small intestine, amino acids rather than peptides appear in the portal circulation. Difficulty may always be expected, however, when we try to show that 100% of any foodstuff follows the principal digestive and absorptive route.

The liver clears a large part of the extra amino acids from the portal blood; a portion of these retained by the liver are gradually released, although certainly not in the same pattern received. The liver therefore serves to modulate the entry of the amino acids into the general circulation.

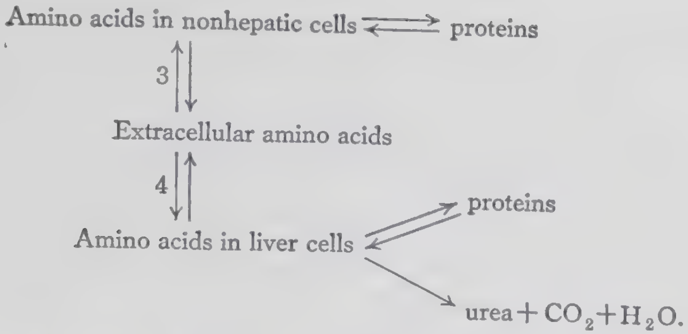
Amino Acid Anabolism vs. Catabolism. The unusual aspect of amino acid metabolism is its strong tendency to swing from an *anabolic* (pathway 1) to a *catabolic* fate (pathway 2).



In infancy, for example, a major part of the dietary amino acids enter protoplasm and a minor part enter catabolism. In adulthood *all* ingested amino acids undergo net catabolism (although some amino acid molecules take a tortuous course to catabolism!). Catabolic swings, drawing away amino acids already built into protoplasm, occur as a consequence of trauma or febrile illness; swings in either direction may be produced by appropriate hormones, and the shifts in general are attributed to endocrine changes.

Van Slyke and Meyer almost a half-century ago showed that the amino acids are not uniformly distributed through the body water but are *at much higher levels in the cells* than in the plasma. Nevertheless the amino acids move freely into the cells, *against* concentration gradients. In fact the greater the growth rate (and hence the faster the flow into the cells), the greater is the gradient! This interesting concentrative process has received considerable study but is not yet fully explained. Account must be taken of it to understand amino acid distribution.

The liver, as we have mentioned, carries out most of the net deamination of amino acids. Therefore catabolic effects may be produced by directing the amino acids *toward the liver;* for example, by intensifying the *hepatic* concentrative activity for the amino acids. Our scheme now becomes a little more complicated:


Amino acids in nonhepatic cells
proteins
3
Extracellular amino acids
4
proteins
Amino acids in liver cells
urea + CO_2 + H_2O.

If such a steroid as hydrocortisone were to stimulate the concentrative step 4, the *catabolic* effect of this hormone could be explained because the amino acids would be exposed at higher levels to the catabolic enzyme systems of the liver cells and thus drawn away from anabolic fates in the rest of the organism. The acceleration of albumin synthesis and gluconeogenesis by hydrocortisone could also be explained in this way, since these reactions also proceed from the hepatic amino acids.

Such effects of hydrocortisone have been shown (Noall, et al.; Riggs, et al.), both for the normal α-amino acids of body fluids and for a model amino acid, α-aminoisobutyric, which escapes catabolism so that changes in its distribution are more clearly observed.

Similarly, intensified concentrative activity favoring other tissues (for example, the uterus upon estrogen administration) may serve to explain growth-promoting effects.

At the same time other possible explanations for the catabolic and growth-promoting effects of these hormones continue to deserve consideration (see Chap. 17).

Interpretation of Plasma Amino-Acid Levels. The interpolation of a concentrative process between the extracellular amino acid and the cellular processes that utilize it, greatly modifies the interpretation of plasma analyses. Occasionally, depressed plasma amino-acid levels are interpreted as representing tissue depletion. From the foregoing, one may instead anticipate lowered plasma amino acids to be associated with *intensified* uptake by tissues. The total quantity of free amino acids in the organism may actually be greater when the plasma amino-acid level is decreased. Conversely, high plasma amino acids may represent *diminished* concentrative activity by tissues.

An interesting example of the operation of the principles underlying amino acid distribution occurs in pregnancy. The total plasma amino acids are diminished by about 25% from early in pregnancy until parturition. Presumably the addition of one more amino-acid-concentrating organism, the placenta (which pumps amino acids into the fetal circulation against a concentration gradient), lowers the maternal plasma amino acids.

Aminoacidurias. A comparatively small part of our amino acids is wasted into the urine, about 150 mg. of α-amino nitrogen as free amino acids and about 250 mg. as conjugated α-amino N. This is usually less than 3% of the total urinary N. This wastage is increased in a number of pathologic aminoacidurias. These are of two types, the *overflow* aminoaciduria, where the defect is prerenal, and the *renal* aminoaciduria, where the disease is one of tubular amino-acid transport.

The former type may be illustrated by phenylketonuria, where the metabolic defect is in the conversion of phenylalanine to tyrosine, the hepatic L-phenylalanine oxidase being absent. As a consequence phenylalanine is chronically at high levels in the body fluids and appears in the urine along with large quantities of phenylpyruvic acid and other related by-products derived from phenylalanine. The mental deficiency perhaps may arise from interference by the high phenylalanine level with the cellular assimilation of other amino acids.

Overflow aminoacidurias may occur also in advanced or severe liver disease, again accompanied by elevated plasma levels.

There are far more illustrations of *renal* aminoaciduria, in which the plasma level of the amino acids concerned is not elevated. The following may be cited:

1. *Cystinuria.* This familial defect involves the resorption of four amino acids, lysine, arginine, cystine, and ornithine, which are lost in quantities descending in the order listed. Because of its very low solubility and the consequent urinary stone formation, cystine received attention long before the others. The presence of two separated cationic groups in the amino acid molecule may be

the structural feature that places these four in a single transport family. The tubular transport of cystine has been shown by Dent to be very nearly zero in cystinuria.

2. *Wilson's Disease.* The primary defect in this genetic disease appears to concern copper metabolism, perhaps arising from the absence of the copper protein, ceruloplasmin. A generalized renal aminoaciduria is also found in most of these patients. They also absorb copper abnormally well from the intestine. Conceivably the increased renal copper load interferes with tubular amino acid transport through its chelating activity; there is also some evidence that metal-chelation may be involved in amino acid transport. Amino acids that are strong chelators and that escape metabolism, can be used to stimulate copper excretion, as can other chelators. The renal defect in Wilson's disease includes decreased inulin and PAH clearance and decreased uric acid reabsorption; hence the aminoaciduria probably should not be overemphasized. Conceivably, ceruloplasmin participates in a mucosal blockade against excessive copper absorption in a manner analogous to the hypothetical operation of ferritin to control iron absorption (Scheinberg). This analogy would have more value if the mucosal iron blockade were better established and understood. On the other hand, injected (native?) ceruloplasmin so far has failed to correct the anomalous response of blood copper to Cu^{64} feeding. The effect on *net* copper absorption is not yet known.

3. *The Fanconi Syndrome.* This is one of the tubular deficiencies mentioned in Chap. 5. In addition to electrolyte disturbances, resistant rickets, and chronic acidosis, these patients show greatly increased urinary losses of most of the amino acids. The disease is inherited as a Mendelian recessive character. Normal plasma amino acid levels and increased amino acid clearances have been shown (Dent) and a morphologic abnormality of the renal tubule recognized (Darmaday). An unexplained and dangerous associated difficulty is *cystinosis,* a widespread deposition of cystine crystals throughout the tissues, which may indicate the presence of a more widespread disturbance of amino acid transport or of some other enzymatic abnormality.

Other tubular deficiencies that result in aminoaciduria have been described. Renal aminoaciduria occurs in congenital galactosemia, in rickets, scurvy, and in poisoning by several agents.

Methodology. Methods for amino acids deserve special comment. Because an aminoaciduria may involve perhaps only one amino acid, the total amino acid excretion may not be appreciably increased. Until the last dozen years, discovery of abnormal excretion products has usually been accidental, as for cystine in cystinuria. Searches for *unsuspected excretion products* are now made systematically by countercurrent methods, especially chromatography on paper or on resin columns. Ninhydrin usually serves for localizing amino acids on such chromatograms; for other compounds a number of reagents have been ingeniously designed. Chromatography on paper is occasionally satisfactory for quantitative analysis, but its major contribution lies in such a semi-quantitative screening for miscellaneous substances.

The ninhydrin manometric method of Hamilton and Van Slyke (or modifications of it) is by far the most satisfactory method for measuring the total amino acids of plasma or urine. Under suitable conditions CO_2 is released from the carboxyl group only when both the carboxyl group and the amino group are free and on the alpha carbon. The older nitrous acid or colorimetric procedures give substantially higher values and are probably not specific enough for blood or urine. The plasma rather than the serum should be analyzed, since the proteolysis associated with blood coagulation releases small quantities of amino acids.

Chemical methods for individual amino acids are in some cases fairly simple, in others complicated. When analysis for several amino acids is desired, resin-column chromatography becomes an attractive procedure. Microbiological assays for amino acids have also been useful, but the results are more liable to artifact.

Alkaptonuria. This rare defect in tyrosine metabolism occurs beyond the transamination step, so that the metabolic intermediate that overflows is not an amino acid but 2,5–dihydroxyphenylacetic acid (homogentisic acid). The presence of this substance in urine causes the urine color to darken and finally turn an intense black. Except for a tendency to develop osteoarthritis the patient is little troubled by the defect. Gradually the cartilage and ligaments become quite dark, a condition known as *ochronosis.*

Coeliac Disease. The ingestion of wheat protein, specifically of gliadin, has been shown to cause anorexia, vomiting, and steatorrhea in coeliac patients. Gliadin has an unusual glutamine content of 43%. If the protein is boiled briefly in N HCl, it becomes harmless (Van deKamer and Weijers). This hydrolysis presumably converts the glutamine residues to glutamate residues without producing extensive hydrolysis of peptide links. Free glutamine is not harmful; accordingly, a difficulty in handling bound glutamine has been proposed. If this proposal is correct, the metabolic difficulty is a unique one in the opening of a common, not very strong linkage.

Peptide Distribution. Our cells contain remarkably small amounts of amino acid conjugates below protein size. Some of these are undeniably peptides, but these peptides are mainly specialized structures like glutathione, anserine, and carnosine. Free peptides of various sizes probably do not serve as intermediates in protein synthesis; instead it appears that the intermediates do not tend to be stripped from the catalytic surfaces until they reach certain degrees of completeness.

Accordingly it is not strange that the plasma contains very little conjugated amino acid which could be intermediate between the proteins and amino acid in molecular size. If commercial preparations of partially hydrolyzed protein are infused, the peptides are more slowly removed from the circulation than are the free amino acids, and large quantities are lost into the urine. At least part of this difficulty in utilizing peptides arises, however, from the generating of antagonists by interactions during autoclaving between the peptides and sugars contained in the preparations. The only proved advantage in having peptides present in amino acid preparations for intravenous nutrition is the technical simplification in their manufacture.

Well over one-half the amino acids in the urine are in conjugated form, but a major part of these conjugates are (like hippuric acid) not peptides. The peptide composition remains to be determined.

LIBRARY PROBLEMS

1. The levels of the various small nitrogen compounds are frequently reported in milligrams of N per 100 ml. of plasma, or in the urine in milligrams per day, originally to permit comparison with the NPN and total nitrogen, respectively, and with the levels of other nitrogen compounds. Such comparisons are no longer made frequently, but perhaps we shall be afflicted indefinitely with the confusion as to whether it is the patient's *urea* or *urea N* that is 20 mg. per 100 ml., for example. When a plasma filtrate is treated with ninhydrin, is *all* the amino *acid nitrogen* released? Is there any method that yields the *total* amino acid N? In the manometric method of Hamilton and Van Slyke, is it actually the α-amino *nitrogen* of the amino acid that is *measured?* Would it not perhaps be an improvement to report the amino acids in millimoles per liter? Is the method perfectly specific to α-amino acids?
2. Examine a table giving the detailed amino acid composition of plasma. Which are the most abundant amino acids? Do the units used permit you to compare rationally the amounts of the amino acids and to calculate the percentage each represents of the total α-amino acid nitrogen? Would not *millimoles per liter,* for example, be a more valuable unit? Are the amino acids that are at the highest concentration those which are passing most rapidly through the plasma?
3. Does the term *nonessential amino acid* mean that the amino acid is not needed for protein synthesis in the cell? Examine the list of amino acids found essential for the growth of cells in tissue culture. Is the list of essential amino acids for a single type of cell apt to be *longer* or *shorter* than the list for the whole organism? If you encounter a paper where you feel that the distribution of the essential amino acids is unduly emphasized at the expense of the nonessential, cite it.
4. Can you find evidence as to the locus of the breakdown of serum albumin? A paper by Loftfield shows that when labeled leucine is administered to the rat, the free leucine in the liver remains at comparatively low radioactivity, although exogenous amino acids readily enter the liver cells. How can this finding be interpreted? Is evidence available as to whether the mammary gland forms milk protein from the plasma proteins or from the free plasma amino acids?
5. Report a paper showing increased copper absorption in Wilson's disease.
6. Report on evidences for disturbances in ceruloplasmin metabolism in nervous or mental diseases other than Wilson's disease. Have such changes been observed by all investigators?
7. Report on efforts to forestall defective mental development by minimizing phenylalanine accumulation in phenylketonuria.

8. Why are sugars ordinarily included in protein hydrolysates to be used for intravenous nutrition? Why is the development of intravenous nutrition by emulsified fat important to the success of parenteral amino acid nutrition? Report on a recent experimental assessment of the success of extensive intravenous nutrition.
9. Examine case 43151, *New England J. Med.*, **256**, 703 (1957). To what chemical substances are many of the symptoms of this patient attributed? From what metabolite is it produced? Why may pellagra-like symptoms result from its accelerated production in carcinoid? What characteristic excretory product results in this disease? This disease was reviewed by Sjoerdsma, et al., in 1957. What urinary test is proposed for screening purposes?
10. Walshe has recently shown that the rate of copper excretion in Wilson's disease can be increased moderately by feeding the amino acid penicillamine. Examine this report and determine why the less expensive cysteine should not be equally good. Propose another possible method of dealing with the positive copper balance of Wilson's disease.
11. Find and report a 1954 paper describing a defect in amino acid transfer by the placenta in toxemias of pregnancy.
12. Disorders associated with the metabolism of phenylalanine and tyrosine were reviewed in 1958. Report on this or a subsequent review.
13. An important method of determining whether intravenously injected amino acids have served their nutritional purpose is the nitrogen balance method. Can you decide from the following data whether this purpose was served in the present instance?

DAY	1	2	3	4	5	6	7
N output, g.	8	Surgery	22	22	19	16	14
N intake, g.	9		6 *	12 *	12 *	12 *	12 *

* Protein hydrolysate.

If not, indicate what control experiment you would need to draw a conclusion. Why does the nitrogen balance method tell how the infused amino acids have been used? Discuss the possibility of determining the balance, not for all the amino acids collectively but for one specifically; for example, the *lysine* balance.

14. Find and discuss a recent report on a hereditary glycinuria. Is it an overflow or renal aminoaciduria? What indication is obtained as the specificity of renal tubular glycine resorption?

12

DISTRIBUTION OF OTHER SMALL NITROGEN COMPOUNDS

Concerning the Nonprotein Nitrogen Method. When blood or serum is treated with sodium tungstate and sulfuric acid in balanced amounts, or with trichloroacetic acid, or with any of a number of other deproteinizing reagents, *very nearly all* the protein is precipitated. The amount remaining in solution, however, is neither entirely negligible nor constant from reagent to reagent. With over 1000 mg. of protein N per 100 ml. of serum and perhaps 30 of nonprotein N, a *failure* of deproteinization can cause an enormous error. We cannot dismiss this hazard, but the signs of a failure are rather obvious.

If now the nearly protein-free filtrate is digested with boiling sulfuric acid and a catalyst, *most* of the nitrogen is converted to NH_4^+, which upon cooling may be measured photometrically as its yellow complex with the $HgI_4^=$ ion in the Nessler technique. Thus the nitrogen of a heterogeneous group of compounds is brought into one form.

Our red blood cells have far lower NPN contents than our other more lively cells; in fact the erythrocytes have only a moderately higher NPN concentration than the plasma (higher mostly because of the cellular glutathione). Therefore this is one of the several cases where we can save the time needed to centrifuge the blood sample and, instead, analyze the whole blood. The normal range of NPN values applying for serum is, however, shifted moderately upward for whole blood. For diagnostic purposes, blood NPN values above 45 mg. per 100 ml. are considered pathologic.

The NPN is more an *analytical* than a *physiological* entity. The analysis is rapidly being displaced by the blood urea analysis, with the advent of simple colorimetric procedures for urea. The factors to be discussed subsequently, which elevate the blood urea N, are also the main ones that elevate the NPN. If the NPN determination is abandoned, much of the historic tendency to consider these small nitrogen compounds together will probably disappear.

Urea. This solute is unusual in that it appears to distribute itself uniformly throughout the body water. The blood concentration apparently is dependent only upon the relationship between its production and excretion, disregarding the minor amount that is destroyed by microorganisms in the alimentary tract. The blood urea N ranges from 6 to 22 mg. per 100 ml., varying directly with the nitrogen intake, according to Wootton, King, and Smith. For our ordinary purposes we should be happier if the *excretory rate* were the only one reflected in the blood level, but even in fasting blood, the normal range is badly spread by the variation in protein intake.

A second factor contributing to the wide range of the normal level of blood urea arises from variation in urine volume. Because urea diffuses back through the tubular cells, and because the gradient is larger and the time for back-diffusion is greater when the urine volume is small, the rate of urea removal or clearance from the blood rises with the urine volume. Therefore persons who eat well but take minimal water contribute to the spread of the extreme upper limit for "normal" blood urea N. If the patient is dehydrated, the urea clearance will be further lowered as the glomerular filtration rate falls.

The latter fact also means that pathologic elevations of the urea N level are readily produced by dehydration. Whether the NPN or the urea is measured, *azotemia*, often severe, is a regular finding in dehydration. In intestinal obstruction, for example, the azotemia may be striking. When there is severe bleeding into the alimentary tract, the azotemia caused by lowered plasma volume and that occasioned by the high protein "meal" are not readily separated, but experiments where blood has been fed show that the former is apt to be the larger factor (Young).

Increases of the urea N (or the NPN) that are clearly out of the broad normal range in a well-hydrated patient point to renal disease.

The analysis is frequently used for screening for indications of kidney disease and for following therapy (for example, sulfonamide therapy) where the appearance of a rise in the urea level is significant.

The Urea Clearance. This rather easy elaboration of the blood urea determination shows how well the kidney excretes urea. Two variables, the urine flow per unit of time and the blood urea level, are taken into account in the method.

To begin with, at a given rate of urine formation the kidney removes a certain fraction of the urea from the blood passing through it, regardless of the blood urea level within a reasonable range of renal blood flow. Rather than stating what *percentage* of the urea is removed from the blood per unit of time, the custom is to state the hypothetical *volume* of blood that is cleared completely of urea in a minute. Van Slyke and his collaborators set this volume at 75 ml. per minute, provided that the urine flow was over 2 ml. per minute. They concluded that the *clearance* became independent of the rate of urine formation above this rate of urine flow, which they called the *augmentation* limit. Later investigators have concluded that the clearance continues to rise with urine flow up to maximal water diuresis.

At lower flow rates Van Slyke's school found that "clearance" with a stable value of 54 ml. per minute was obtained if the usual clearance equation

$$C = \frac{U \cdot V}{B}$$

(where U = urine urea concentration, B = blood concentration, and V = milliliters of urine per minute) was replaced by the empirical equation

$$C_s = \frac{U \cdot \sqrt{V}}{B}$$

The term *standard clearance* (C_s) was used for this arbitrarily calculated quantity. The use of the square root of the urine minute-volume has no apparent physiological basis but is only a working approximation. Tables have been constructed for "correcting" the urine volumes of children so that the same normal clearances (±20%) may be anticipated for them.

One would not select the urea clearance test for physiological research because no single renal function (for example, glomerular filtration) is isolated. Nevertheless the test rather simply adds a great deal of precision to the unaccompanied blood urea determination. In practice the two main sources of error lie in *timing the test* and in *measuring the urine volume.* If it is understood by the personnel supervising urine collections that the period of each collection need not be exactly 1 hr., but must be accurately known, better results will be obtained. Also the urine samples are usually best sent to the laboratory in their original vessels, the volume to be measured there.

Creatinine. Creatinine is produced at a rather steady rate as a consequence of a spontaneous instability of creatine phosphate. This rate presumably depends upon the body content of creatine phosphate and indeed has been linked to the muscle mass of the subject.

Creatinine (in contrast to creatine) is rarely determined to investigate creatine phosphate metabolism, but instead, as for urea, it is used as an indicator of renal retention. The blood level of creatinine is subject to less normal variation than the urea level. The creatinine clearance has theoretical advantages to the urea clearance in many animals in that it corresponds with the glomerular filtration rate. In man, however, additional creatinine is excreted across the tubular cells. As in the urea clearance the endogenously present creatinine usually serves for the test; in either case this procedure has the advantage that the plasma level is nearly constant over many hours, which is not true when the clearance of an *administered* substance is measured, unless it is continuously infused. The creatinine clearance is less dependent upon the urine volume than the urea clearance and in practice is a much more satisfactory measure of renal function.

Uric Acid. This substance is the end product of the catabolism of the purines we eat or synthesize. The main object of uric acid analyses, however, has not been to study purine metabolism. Occasionally, increased uric acid excretion is used to measure the accelerated release of nucleic acids in the breakdown of reticuloendothelial cells; for example, during adrenocortical steroid treatment or in the treatment of leukemia. Conceivably, a differential analysis of urinary purines and pyrimidines may one day provide a differential diagnosis for the leukemias. Usually, however, uric acid is determined for purposes unrelated to purine metabolism.

A large, often major, part of the uric acid arising from our metabolism comes from dietary purines. Endogenous uric acid, from the oxidation of the purines derived from our own nucleic acids and nucleotides, may represent about 0.5 g. per day. The biological synthesis of the purines has been worked out in brilliant fashion (Buchanan, G. R. Greenberg). Figure 12.1 shows the origins of the portions of the molecule from glycine, formate, CO_2, and (for N atoms) from glutamine and aspartate. The assembly occurs largely in the ribotide forms, i.e., with a ribosephosphate residue attached at nitrogen atom number 9. The other purines can be formed from adenine, and all are subject to eventual oxidation to uric acid.

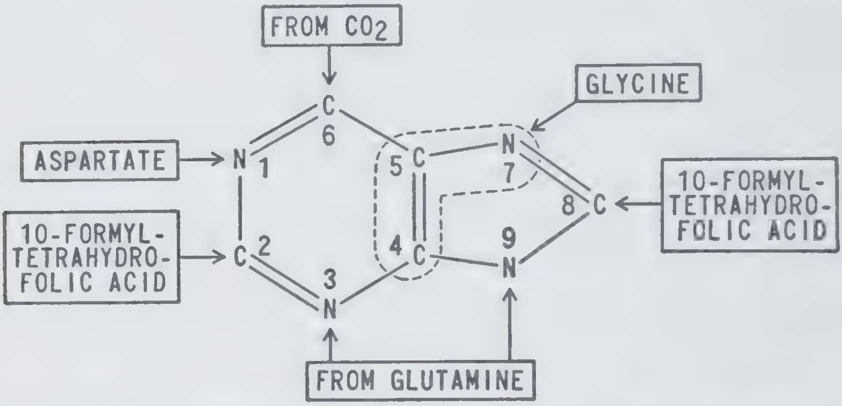


Fig. 12.1 Biosynthetic origins of portions of the purine molecule. (See reviews by Buchanan, et al., and by Greenberg and Jaenicke, in Ciba Foundation symposium on *Chemistry and Biology of Purines,* London, 1957.)

Conceivably, portions of the purines formed synthetically are oxidized to uric acid without ever entering nucleic acids. In general when a substance serves in the cell as a stage both in anabolism and catabolism, we may anticipate that some molecules of the synthetic intermediate may come in contact with a catabolic enzyme before it encounters the next enzyme in the anabolic system. The apparent undesirability of such destruction does not, of course, constitute a shield against the degradative enzyme. Suitable spatial localization of enzymes may serve to diminish the diversion of intermediates from specific pathways.

From the extent to which an injected dose of labeled uric acid is diluted, Stetten and his associates calculated that the body contains about 1.1 g. of uric acid (presumably present as the urate ion). About one-sixth of this is present in the blood, five-sixths outside the blood stream. These figures do not suggest that the cells contain appreciably higher levels than the extracellular fluid. No evidence of protein-binding has been detected in the blood. About one-half of this uric acid is normally eliminated and replaced each day. A significant fraction is destroyed, either by intestinal microorganisms or in the tissues.

Gouty patients have moderately higher serum uric acid levels than normal persons. Jacobson found levels below 6 mg. per 100 ml. for 97 of 100 normal individuals, whereas in 21 cases of gout the range was from 5.2 to 14.8 mg. per 100 ml. In 98% of his determinations in gout, the level exceeded 6 mg. per 100 ml. These figures obviously mean that the analysis is a rather effective discriminator. The *body content* of uric acid (as shown by isotope dilution) in gout is more strongly increased than the *serum level*, from 3 to 20 times normal (Stetten). Obviously, therefore, this *extra* uric acid is not uniformly distributed through the body water. The precipitated deposits characteristic of the disease are assumed to account for most of the extra uric acid. Furthermore, the amounts present in this precipitated form are apt to be underestimated by this procedure because of slow exchange of the crystalline sodium urate with the administered labeled uric acid.

Either such patients form uric acid more rapidly than normally, or they excrete it more slowly. The experiments of Stetten and of others indicate that it is the *synthesis* which is faster. Late in the disease, renal involvement also leads to slower excretion. Studies undoubtedly will be forthcoming to show which reaction step is specifically accelerated. Conceivably a purine intermediate in nucleic acid synthesis in the gouty patient is less adequately protected from the catabolic systems than normally. In birds and reptiles a major portion of the synthesized purines passes directly to uric acid. The finding of uric acid riboside, and an enzyme transferring its ribose to phosphate in human tissues (Laster), adds an additional possible site for the metabolic defect.

Hyperuricemia also occurs during gross tissue destruction of various origins, in which case the uric acid presumably arises from nucleic acids and nucleotides. Local factors must contribute to the formation of urate deposits in gout, since levels of uric acid artificially maintained above the solubility of sodium urate do not lead to attacks.

Uric acid accumulates in plasma along with urea and creatinine in renal disease; in toxemias of pregnancy the accumulation is comparatively specific and considerable prognostic significance is attributed to the analysis (Peckham).

Both uric acid and the urate salts are comparatively insoluble, so that the urine is regularly supersaturated and crystals of various types are found in urinary sediments according to the urine pH and composition. Stones of these compositions occur occasionally in the urinary tract.

Plasma Ammonium Ion. This substance is an excretory product in the urine, where it appears at the expense of urea N, as discussed earlier, to an extent determined by the acid-base balance. In tissues and in the blood plasma, however, the ammonium-ion concentration is very low and has quite a different significance.

A principal source of metabolic ammonium ion is the amino group of *glutamate* through the action of glutamic dehydrogenase (see Fig. 8.2). This reaction accounts for a large part of the net deamination of amino acids because amino groups are transferred from the various other amino acids to α-ketoglutarate to form glutamate. Several amino acid oxidases also release smaller amounts of NH_4^+ directly from various amino acids. The released ammonium ion can re-enter amino acid or aminopurine structures, since these compounds become labeled extensively when N^{15} ammonium citrate is fed. Therefore NH_4^+ can serve as an intermediate in the transfer of amino groups among amino acids and other compounds, although a much larger part of such transfers occurs by transamination, i.e., without release as *free* NH_4^+.

A considerable amount of intermediate formation of NH_4^+ undoubtedly occurs, at least in the liver, in normal metabolism, probably largely from glutamate, the NH_4^+ going largely to form citrulline in the urea-synthesis cycle. A portion is tied up reversibly in the form of glutamine. This organ can tolerate a considerable concentration of NH_4^+ arriving in the portal blood after NH_4Cl feeding. The liver fixes this NH_4^+ almost quantitatively, so that only a very small concentration enters the systemic circulation. Probably NH_4^+ arises in the lower intestine rather constantly through bacterial action, so that this ammonia-fixing activity goes on continually.

Other tissues, however, tolerate only very low concentrations of circulating NH_4^+. This metabolic intermediate is most likely too reactive to be tolerated at increased concentrations. The resultant metabolic disturbance is especially important in the central nervous system, if we may judge from the tendency of hepatic coma to occur when the blood ammonia has been elevated. Hepatic coma is described as an episodic stupor accompanied by a flapping tremor.

Ammonium ion is believed to reach the systemic circulation in liver disease mainly because of the formation of collateral circulation by which portal blood by-passes the liver. Dogs with an Eck fistula have long been known to be susceptible to *meat intoxication*. Similarly coma can be produced by feeding NH_4^+ salts or high-protein diets to patients with surgical or spontaneous shunts past the liver. Nonabsorbable antibiotics given by mouth combat ammonemia and coma in patients with liver disease.

Poor agreement has usually been reported, however, between the blood NH_4^+ level and the degree of coma observed in liver disease. The agreement has been reported to be better if the NH_4^+ of the *arterial* blood is measured, the venous level representing the NH_4^+ left after NH_4^+ fixation by the tissue that the venous blood has perfused (Bessman). Others report the correlation poor even for arterial ammonia. Another suggestion is that NH_4^+ enters cells as NH_3. Although only about 1% of the NH_4^+ is in this form at pH 7.4, the NH_3 pressure changes rapidly with a pH change, being only one-half as great at pH 7.1. If NH_3 is the form transferred, then the P_{NH_3} may be decisive.

Finally the NH_4^+ may well disturb metabolism by depleting another cell constituent, which means that variations in the rate of formation of that constituent can also modify the effects of a given increase in the ammonia level. Glutamate has frequently been suggested as the substance depleted in brain tissue by the glutamine synthetase reaction

$$\text{Glutamate} + NH_4^+ \rightleftharpoons \text{glutamine} + H_2O$$

Glutamate therapy has not given uniformly good results, however, although this may mean that its administration fails to modify the brain glutamate level. Of course glutamate may serve as an NH_4^+ *source* by conversion to α-ketoglutarate as well as a fixer of NH_4^+. The metabolite, if any, whose concentration in the brain may be critically modified by elevated plasma NH_4^+ cannot yet be said to be identified.

Poor uptake of NH_4^+ by peripheral tissues in liver disease has been reported by Summerskill, Wolfe, and Davidson. They find the blood pyruvate and α-ketoglutarate to be *elevated* in hepatic coma; these concentrations are further increased by the administration of NH_4Cl. Conceivably the prior NH_4^+ load has handicapped NH_4^+ fixation.

Arginine Stimulation of NH_4^+ Fixation by Liver. A source of toxicity during the intravenous infusion of amino acid mixtures is NH_4^+ formation, part of this NH_4^+ being released into the circulation. Increasing the arginine content of the mixture can largely eliminate this toxicity and prevent the rise in the blood NH_4^+ (Greenstein). The protective action of the arginine in animals increases as the site of injection is chosen to bring as much as possible of the dose directly to the liver; for example, by injection into the spleen. Arginine or ornithine administration is also effective in lowering the blood NH_4^+ in liver disease (Najarian) but rather irregularly useful in alleviating hepatic coma (Wolfe).

Clearly the diagnostic value of the blood NH_3 determination is severely restricted because the concentration measurement fails to separate the *influx* of NH_4^+ into the blood, from the intestine and various tissues, and the *removal* of NH_4^+ by various tissues. As an added difficulty we are, of course, dealing with chemical events that do not occur in the blood stream.

The Analytical Problem. The normal blood ammonia level usually lies between 0.05 and 0.1 mg. per 100 ml. Shed blood, however, rapidly forms ammonia from adenosine and other substances, this release being stimulated by the K_2CO_3 often added in the analysis. Consequently, analytical values have varied from laboratory to laboratory and have frequently been considerably higher than the above values. This difficulty appears to have been obviated by the finding that immediate trichloroacetic acid deproteinization at ice temperature stops NH_4^+ release. The resultant filtrate is said to be quite stable and leads to consistent results (Nathan and Rodkey). Why can this simple step of deproteinization be so effective in preventing artifactual ammonia formation?

LIBRARY PROBLEMS

1. Compare tables from various sources giving the normal ranges for whole blood and serum NPN, urea N, α-amino acid N, and uric acid. Why do the figures vary? Propose a standard statistical basis for setting the normal range of a biological variable. When is a carefully selected basis for setting the limit between normal and pathological values most important? Calculate how much a very low hematocrit can reduce the upper limit of normal for the whole blood NPN.
2. Consider the likelihood that "toxic destruction of protein" can account for a large rise in blood NPN or urea in disease. Can you find analyses to show that large quantities of intermediate protein breakdown products enter the blood in disease? A patient discussed in case 44171, the *New England J. Med.*, **258**, 864 (1958), was calculated to be losing a pint a day of blood into the intestine, and yet her NPN was only 25 mg. per 100 ml. Does this contribute evidence as to the origin of azotemia in gastrointestinal bleeding?
3. The fact that urea appears to be uniformly distributed throughout the body water suggests its possible use for measuring the volume of the body water. What disadvantages does it have for this purpose? What substances have been used for this purpose and with what results?
4. Find and report one or more papers appearing since 1950 that show whether the creatine appearing in the urine in progressive muscular

dystrophy arises from muscle or is instead synthesized elsewhere but fails to gain access to the muscle. Consider various factors that may account for creatinuria.

5. Look up the use of a specific enzyme for the determination of serum uric acid; for creatinine. Did these analyses establish that the ordinary colorimetric methods are sufficiently specific? Look up the pK's applying for uric acid. What form should be present at pH 7.4? At the lowest physiological urine pH?
6. Consider on the basis of reading the question whether uric acid is further degraded by tissues of the human or animal organism.
7. Blood ammonia analyses are included in cases discussed in the *New England J. Med.*, **253,** 737 (1955); **254,** 297 (1956); **255,** 337 (1956); **255,** 1243 (1956); and **258,** 190 (1958). Which of the reported analyses were elevated? How were the results related to the presence or absence of coma? What other chemical findings were abnormal in the last-mentioned case? Glutamate therapy was used in a case presented in *New England J. Med.*, **253,** 737 (1955).
8. The discussion of case 42051, *New England J. Med.*, **254,** 226 (1956), includes the question of the origin of the low blood urea levels of patients with liver disease. The suggestion was made that perhaps part of the patients' food nitrogen was being converted to ammonia N in the blood. Does this sound plausible to you? In case 42461, *New England J. Med.*, **255,** 958 (1956), consider a question by Dr. Brailey who asks whether red-cell breakdown would lead to uric acid formation.
9. On the basis of reading recent papers from Greenstein's laboratory, discuss the mode in which administered arginine antagonizes the release of ammonia in toxic amounts. Does a finding that injecting arginine increases the blood urea show that it is functioning by converting blood ammonia to urea?
10. One occasionally reads that although the human renal tubule contributes some creatinine to the glomerular filtrate, this source of error in estimating the glomerular filtration rate is compensated by the usual *overestimate* of the serum creatinine arising from the presence of substances other than creatinine in the serum, which give color in the creatinine method. Do you think this re-establishes the creatinine clearance determination in man as a measure of the glomerular filtration rate? What other substance may serve for this measurement?

13

CIRCULATING PROTEINS: NORMAL AND ABNORMAL

The protein molecule is composed of one or more peptide chains, usually with somewhat specific orientation among the chain parts. An early reaction to the complexity of these molecules was to suppose that their properties would not bear the usual relationships to their composition, that a separate science of *macromolecular chemistry* would be necessary. Beginning with the classical studies of Jacques Loeb, it has instead become apparent that the behavior of proteins will be entirely explained by their structure, and that between micro- and macromolecules, there is only added complexity and no chemical discontinuity. The dissociation behavior for example of side-chain amino and carboxyl groups shows no particular difference in small peptides on the one hand and in complex protein molecules on the other. Powerful chromatographic and electrophoretic methods are advancing concurrently for separating micro- and macromolecules.

The ready metabolic interconversion of micro- and macromolecules likewise means that they are closely correlated in biological significance. A greater discontinuity lies perhaps in the curious scarcity of related molecules of *intermediate* size as tissue constituents. In the *blood plasma,* more than in our *cells,* there is a considerable difference between the significances of the protein and the micromolecular components.

The Total Plasma Protein. Present methods make this a very simple determination. In a standard laboratory procedure 0.1 ml. of serum is added to an alkaline copper sulfate solution and the purple "biuret" color is measured photometrically. Where a photometer is not available, an even simpler but slightly less accurate procedure is the *sitting-drop* method of Lowry and Hunter, which is much more trouble-free than the older falling-drop procedures. The method involves the use of a cylinder containing two different mixtures of bromobenzene and kerosene (for example), a dense mixture in the bottom, and a less dense one on top. A gradient that will persist for many months is formed by controlled partial mixing. A drop of the serum falls through such a column until it reaches a level of its own density. The position taken can be compared with the positions taken by K_2SO_4 solutions of standard density equivalent to known protein levels. Since the principal variable factor in contributing a density above 1.00 to serum is the protein concentration, a fairly accurate measure of the total serum protein level is obtained in a few seconds.

This measurement of all the proteins together is of limited value because of the great difference in the significance of various proteins. It is mainly valuable as a screening method for hypo- and hyperproteinemias, which can then be studied in detail, and as a method for following the progress of a patient (especially a surgical patient) from time to time. Also the determination of total serum protein is a part of several of the methods for the separate determination of albumins and globulins.

Precipitation of the Globulins for Separate Analysis of Albumins and Globulins. Starting with pure water as the solvent, proteins characteristically have their solubilities *increased* by the addition of electrolytes, but as the salt concentration rises still higher, the solubilities pass through maxima and then *decrease* again. The proteins whose solubility curves lie so low that the solubility in pure water is considered negligible are by classical definition *globulins.* Frequently these are also nearly insoluble at a salt concentration corresponding to *half-saturation* with ammonium sulfate. A second definition of globulins involves this second criterion, namely, insolubility at the half-saturation level of ammonium sulfate.

These two definitions are inconsistent for the *pseudo-globulins,* which meet the *second* test but not the *first, more classical* one.

P. E. Howe in 1921 chose Na_2SO_4 for this precipitation of globulins to avoid the interference of ammonium sulfate with analytical procedures. Apparently through a misinterpretation of his own results, he chose a 22% concentration of the salt, although later work has shown that concentration of about 27% is necessary to complete the precipitation of globulins. As a result the globulin precipitate is too small, and analysis of the filtrate from this precipitate gives a falsely high result for albumin. Extensive experience with the electrophoresis of samples from large pools of plasma have shown that albumin represents about 55% of the normal plasma protein, corresponding to a ratio of the albumin to the globulin concentration of 1.2. Such a value by the Howe method would be regarded as pathological, since by this method an average of about 64% of the protein is found in the "albumin" fraction, corresponding to A/G = 1.8.

Clearly this is a very serious difference, and one may be tempted to say that the Howe method should be abandoned. Modern methods (for example, methanol precipitation at low temperatures according to Pillemer and Hutchinson, or the use of 28% sodium sulfite) give results corresponding much more closely to the electrophoretic findings, and undoubtedly with experience the results of such methods will be as useful as the results by the Howe method are today. Such a wealth of experience has been gained with the results of the Howe analysis, however, that there is no question of its utility. The main difficulty is that its empirical results are stubbornly labeled *albumin* and *globulin*, leading to confusion upon comparison with more correct analyses. Published normal standards for the serum albumin and globulin levels must be labeled as to whether they apply for this Howe method or for another procedure. Similarly the method used in securing any diagnostic result for albumin and globulins must be known.

The difficulty is intensified if the concept of the *inversion* of the albumin to globulin ratio is accepted. In liver disease or in infections, the albumin level tends to fall and the globulin level to rise. If the tendency is severe enough, the Howe ratio becomes less than one, and the ratio is said to be *inverted*. Note, however, that if we deal with *true albumin* and *globulin* analyses, the ratio normally averages 1.2, which is so close to unity that the ratio "inverts" very easily without dire significance. Therefore the concept of *inversion* of the value A/G has become ambiguous, and really is useful only when the Howe method is used and specified.

Incidentally, one frequently hears the *A-to-G ratio* requested when the *ratio* is not exactly what is desired. This term seems to be used as a crude shorthand for the albumin and globulin *levels*, as can usually be discovered if one replies with the ratio only. Each of these classes of protein has its own diagnostic significance; knowledge of the relation between them may add supplementary assistance, but such knowledge is not so important as knowing the actual concentrations of each.

Whatever method is used for precipitating the globulins, the usual procedure is to measure the protein left in solution; for example, by the biuret method. The difference between the total protein and the "albumin" is ascribed to the globulins.

Electrophoresis. Even by the more specific methods the globulin analyses give no information about the levels of the numerous individual proteins included in the globulin fraction. These also have special individual significance. By electrophoresis of the serum buffered at pH 8.5 to 8.6 in a very narrow tube maintained at 4°C., the proteins are caused to migrate as anions and may separate from each other because of their different rates of migration. Albumin moves the fastest among the well-characterized classes, although a boundary moving even faster has been observed repeatedly. The globulins follow in the sequence α_1, α_2, β, and γ. After a period of time the forward (or rear) boundary due to each of these protein classes becomes discrete and can be located by an appropriate optical system to produce the *electrophoretic diagram*. The actual concentrations can be obtained from the area under each individual peak in the electrophoretic diagram, with the following average findings for plasma (Armstrong, Budka and Morrison):

ALBUMINS	GLOBULINS			
	α_1	α_2	β	γ
55.2%	5.3%	8.7%	13.4%	11.0%

(The discrepancy from a total of 100% arises from the appearance of 6.5% of the total protein in a "fibrinogen" fraction.)

Electrophoresis *on paper* has been proposed to avoid the expense of the routine clinical use of Tiselius-cell electrophoresis. After migration has occurred, the proteins are caused to adsorb a pigment, and the depth of color is determined by scanning the paper with a densitometer. This instrument yields an electrophoretic diagram from which the amount of each protein can be quantitated. This numerical interpretation (not the colored paper) is, of course, the useful product of the analysis.

The separation of the serum proteins into the above half-dozen fractions is unfortunately still only a crude resolution of the many indivdual proteins present. Diagnostic importance almost certainly will be found for many of the indivdual proteins included in these classes, so that better resolution is much needed. Either *electrophoresis* on *starch blocks* or *column chromatography* upon *modified celluloses* offers the promise of the needed extra resolution. Chemical treatment of cellulose to introduce charged groups has produced adsorbents especially suitable for chromatography of proteins. In the meantime the diagnostic utility of electrophoretic analysis is limited to a modest number of situations where characteristic changes in the major components or fractions may be anticipated.

γ-Globulins. Under electrophoresis the antibodies formed in response to antigen injection or infection migrate mainly, but not exclusively, in the γ-globulin group. Accordingly, preparations of this fraction have found considerable therapeutic use. The γ-globulins are a heterogeneous group for which we still lack ready resolution. Proteins of this class may be almost totally absent from the plasma (agammaglobulinemia) as a congenital disease or as an acquired disease, both arising from the absence of the plasma cells that ordinarily synthesize these antibodies. At least two proteins migrating in the β-class may also be deficient (Gitlin). A transient form of agammaglobulinemia also occurs in infants.

Glycoproteins. Serum contains a considerable number of proteins that contain *stably bound* carbohydrate; these should be designated as *seromucoids.* Another name, *mucoproteins,* should be reserved for a second class of glycoproteins, possibly not present in blood serum, that release their carbohydrate readily; for example, by treatment with strong salt solution or dilute alkali.

The sugar units recognized in the seromucoids are galactose, mannose, fucose, glucosamine, galactosamine, and the complex substance sialic acid. Normal serum contains in its proteins about 0.12% total hexose, 0.083% hexosamines, 0.009% fucose, and 0.060% sialic acid (Winzler). Whether these constituents are present in polysaccharide form or in smaller units scattered over the protein molecule is not clear. These several classes of carbohydrates are probably present in different amounts in the various glycoproteins.

Known examples of these glycoproteins are the acidic orosomucoid of Schmidt, which is an α_1-globulin, also three α_2-globulins, and prothrombin. One of the α_2-globulins contains 4.35% hexose (Surgenor); another is much higher in hexose plus hexosamine. Specific glycoproteins have important thyroxine- and corticosteroid-binding properties (see Chaps. 16 and 17).

Analytical methods are available for protein-bound *hexose, hexosamine,* and *fucose;* two other color reactions give results closely related to the *sialic acid* content. In addition, extraction with perchloric and phosphotungstic acid removes certain mucoids. Undoubtedly older reports of high plasma peptides derive from such solubility of proteins in deproteinizing solutions. Immunochemical methods have been used for individual glycoproteins.

Proteins in these classes tend to be increased in neoplastic disease, tuberculosis, diabetes with vascular disease, pregnancy, rheumatic disease, rheumatoid arthritis, lupus erythematosus, inflammatory reactions, thermal injuries, fractures, and after parathyroid hormone administration. Tissue destruction, or possibly accelerated tissue proliferation, may be the stimulus to their release. Both the liver and depolymerized ground substance have been suggested as sources; the latter ought, however, to contribute *uronic acids* to the serum.

The length of this list of diseases is not necessarily discouraging because it refers to a collective measure of a variety of proteins. The great need in this field, as for the γ-globulins, is for better resolution of the glycoproteins so that the association of a change of an individual protein with a specific disease may be detected. Already there are indications of such specificity (Fahey). The same separation methods proposed under "Electrophoresis" in this chapter are promising. At the moment, however, the diagnostic value of glycoprotein analyses is largely undeveloped.

Metal-binding Globulins. Proteins in general usually chelate with the transition group and heavy metals, but a crystalline β-globulin, *siderophilin,* has a higher affinity of the right order to keep it partially loaded with iron at the concentrations of iron normally prevailing. Two atoms of iron are bound by each protein molecule. The protein also binds zinc and copper, but the affinity for iron is greater, so that there is a question as to whether it operates to transport other metals.

A valuable chemical analysis is the measurement of the iron-binding capacity of the serum, which determines how nearly the siderophilin is saturated with iron. In this analysis a chelator is added that gives a color with iron but which is not so strong a chelator as the metal-binding protein; hence color does not appear until the binding capacity is saturated. (Here is as good an example as any other to test your comprehension of molecular distribution.) Because the affinity of this protein is of the appropriate order, its iron load is responsive to the iron level of the organism, permitting it to be used to gauge the effective iron level of the body fluids. A high degree of iron saturation of the protein is considered practically diagnostic of hemochromotosis.

A blue copper-containing protein, *ceruloplasmin,* which contains most of the circulating copper, was isolated from serum by Holmberg and Laurell. In a molecule of 180,000 weight there are eight copper atoms. This protein releases one-half its copper on reduction to a colorless form by ascorbic acid. Although the copper does not exchange *in vitro* in the oxidized state, this compound can be visualized as possibly serving in copper transport. The protein is enzymatically active in oxidizing amines, methylated *p*-phenylenediamine derivatives being used to assay this activity. Epinephrine oxidation is also catalyzed. The protein has been found absent in cases of Wilson's disease (Scheinberg and Gitlin) and is reported to be elevated in many schizophrenics (Akerfeldt) as well as in a number of unrelated diseases.

The protein may be measured directly in serum in a good spectrophotometer by its blue color, although cuvettes of an unusually long light-path are desirable.

Discussion of the *lipoproteins* will be deferred to Chap. 14.

Serum Albumin. Like most of the serum proteins (prominent exception, some of the γ-globulins), albumin is synthesized in the liver, such synthesis having been demonstrated *in vivo,* in liver slices and in perfused liver. Albumin synthesis and breakdown proceed constantly at equal rates to yield a half-life in man of three to six weeks. The failure to establish unequivocally the locus of the breakdown of the serum albumin is an embarrassing gap in our knowledge. This may well occur in the liver; for example, Loftfield has shown that labeled leucine injected into the blood stream is subjected to an intense, constant dilution in the liver, as shown by an inability to bring the liver leucine to a high specific activity. Accordingly a large nonsynthetic source of endogenous leucine in the liver must be presupposed. Partial hepatectomy slows the removal of radio-iodinated albumin from the circulation of the mouse; reticuloendothelial "blockade" with the substance thorotrast has a similar effect, pointing perhaps to the Kupfer cells (Hughes).

Another suggestion has been made that part of the albumin is broken down in the kidneys. A normal loss of albumin through the glomerulus, followed by tubular recovery and catabolism of the albumin, has been proposed as possibly fast enough to explain the whole albumin breakdown. Ligating the ureters of the mouse produces a moderate slowing of the removal of radio-iodinated albumin (Hughes). Clear-cut evidence for extensive proteolysis in the kidney, however, is still lacking.

A very influential proposal by Whipple and his collaborators attributes to the plasma proteins a function as major transfer forms of nitrogen among tissues. Clearly the plasma proteins *can be ultilized* to form tissue structures, and the tissue proteins *can be utilized* to form plasma proteins. The key questions concern the normal *extent* and the *pathways* of these interconversions.

The investigations of Whipple's laboratory, recently summarized in a compact monograph, show that the dog can be maintained on a protein-free diet if sufficient plasma proteins are infused. The circulating level of plasma proteins in the dog must be brought to 9 or 10 g. per cent, with resultant proteinuria, in order to supply plasma proteins at a sufficiently rapid rate for this purpose. Although the dogs lose a little weight, they are in *positive* nitrogen balance.

Such a positive nitrogen balance was noted also after albumin infusion by Davidson and his collaborators, but it was interpreted instead to represent an even greater accumulation of albumin than the plasma analyses revealed, a large part of the albumin being extravascular. These results agree in showing that a considerable pile-up of plasma proteins occurs, indicating a *fairly tight bottleneck* in the utilization of the plasma proteins.

Whipple and his associates also showed that tissue proteins are consumed to renew the plasma proteins in the dog under *plasmapheresis.* Two grams of nitrogen could be transferred from tissue to plasma protein per day when the plasma protein level was kept down to 4 g. per day by constant operative removal. The ability of the organism to form tissue proteins at the expense of plasma proteins, and vice versa, cannot be questioned.

Whipple further inferred that tissue and circulatory proteins are interconverted *without degradation to the amino acid level.* The evidence for this view is indirect: When plasma proteins were *injected,* there was much less urea production and much less $C^{14}O_2$ release (the protein having been labeled with C^{14} lysine) than when the protein was *fed by mouth.* The oral route does not provide an ideal control for studying the fate of intravenously injected proteins. If we could arrange an experiment so that the amino acids of the *fed* protein could be delivered to the tissues over a period of many days, as undoubtedly occurs after plasma protein *injection,* we should be able to determine how much catabolism to expect for a conversion via free amino acids.

The indirect evidence obtained so far is questioned because in a number of situations where one protein is converted into a quite different one, close study has shown that the *free amino acids* are the intermediates. For example, the plasma proteins were for a long time supposed to be converted into milk proteins. Milk can be secreted so rapidly that one does not readily believe that body proteins pass to milk proteins via the very dilute plasma amino acids, but such has been shown to be the case, using C^{14} labeling. Even in cases where one protein molecule is sacrificed to form another within a single cell, amino acids have been found to be the intermediates. Many experiments have indicated that this occurs with serum albumin (*cf.* Haurowitz, Campbell), but the evidence cannot yet be regarded as unequivocal.

Studies of protein synthesis have led to serious doubt that *free* polypeptides are important synthetic intermediates, which they would need to be for large fragments of one protein to be incorporated extensively into another. This idea has always been attractive for its economy, but no single instance has yet been established where a free peptide enters a protein molecule directly without cleavage. Isolated instances of such transfer may well be discovered, but it appears likely that each incipient peptide chain of the protein molecule generally is completed before it is dissociated from the catalytic surface. This concept is sometimes spoken of as the "zipper" theory of protein synthesis.

Conceivably the albumin must return to the liver for hydrolysis before it leads to new tissue protein.

The implications of the dynamic state of the proteins are in any case probably of full clinical applicability: The plasma proteins are not readily kept up to normal levels in a malnourished individual. On the other hand the possibilities of nourishing the human patient by plasma injection are rather poor because of the relatively unsuitable amino acid composition of the constitutent proteins as well as the lag in the conversion.

Although serum albumin is able to leave the liver cell after its synthesis, and is probably able again to re-enter that cell (and perhaps others) for destruction, we must guard against overestimating the extent to which proteins can be transferred from cell to cell. For example, *phosphorylase* is present in skeletal muscle, heart muscle, and liver; yet for a given species the enzymes present appear to be *different ones at each site,* a fact also of genetic interest.

Because of an unusual internal flexibility in its structure, albumin is able to bind a large number of substances of all kinds. If the substance is a metabolite that is otherwise rather insoluble in water, like free bilirubin or unesterified palmitate, a transport function is attributed to the albumin. For soluble drugs, however, we may look upon the binding as a useless sequestering.

The serum albumin levels may be decreased by *inadequate synthesis,* not only in undernutrition but also in liver disease, or by *excessive loss* into the urine in the nephrotic syndrome or into the intestine, or by exudation in burns, or by inadequate return to the vascular system, as in the ascites of cirrhosis. Hemodilution may also lower the effective level. The primary importance of albumin in maintaining the colloid-osmotic pressure of the blood is reemphasized.

The nephrotic syndrome may arise from a variety of types of renal disease and is characterized by massive proteinuria, edema, hypoalbuminemia and hypercholesterolemia. The glomeruli are unduly permeable to proteins, so that the smaller molecules, especially albumin but also some of the smaller globulins, pass through in unusual amounts. As the serum albumin level falls, its excretion rate is reduced and usually comes to equilibrium with the rate of albumin production. The hypoalbuminemia is usually severe enough to produce edema when the albumin loss is 10 g. or more per day. Under these conditions the rate of synthesis of serum albumin is abnormally high, as shown by the rate of entrance of labeled amino acids into the protein.

Urinary losses of the thyroxine-binding and iron-binding globulins lead to abnormally low hormonal iodine and serum iron levels, without implications as to the thyroid activity or iron economy. The lipemia of nephrosis will be discussed in Chap. 14.

Abnormal Globulins of Multiple Myeloma. One-half to two-thirds of patients with multiple myeloma excrete into the urine *Bence-Jones protein,* a group of related proteins of curious properties. A majority of the patients also have a *hyperglobulinemia,* total protein levels as high as 18 g. per cent or more having been reported. This rise is caused by abnormal globulins usually migrating in the gamma class which, however, are *not* similar to the Bence-Jones proteins excreted in the urine. Whereas the abnormal *plasma* globulins have molecular weights in the neighborhood of 160,000 and contain carbohydrate, the *urinary* proteins are in a 30,000 to 40,000 molecular-weight class and contain no carbohydrate.

The amino acids that form the ends of peptide chains are also entirely different. Labeling methods reveal that the abnormal *plasma* proteins cannot be precursors of the Bence-Jones proteins, since they become labeled comparatively too slowly (Putnam). The opposite relationship is still possible, namely, that part of the Bence-Jones proteins may be converted to the abnormal plasma globulins rather than lost into the urine. The finding that almost every one of 31 myeloma patients had his own individual Bence-Jones protein leads to the possibility that numerous individually characteristic metabolic blocks lead to incomplete protein synthesis within the plasma cells (Putnam and Miyake).

Proteins in Other Extracellular Fluids. An extremely rapid exchange of protein between the vascular and extravascular extracellular fluids has been shown. Apparently a comparatively rapid escape, especially of albumin, occurs from capillaries, with return of the protein by the lymphatic system. Many of the plasma proteins have been identified in the synovial fluid, although the total protein level is much lower than in the plasma.

Plasma Enzymes. The blood plasma certainly contains some functional enzymes or proenzymes (for example, those associated with blood coagulation and fibrinolysis); others are normally present but are not recognized to serve any purpose in the plasma (amylase, phosphatases, esterases, etc.). The latter, along with others that may be normally indetectible in the plasma, are released into the plasma in large amounts as a result of disease in one tissue or another.

The problem of discovering enzyme *concentration* by an assay of enzyme *activity* deserves comment here. One example is the assay of prothrombin known as the determination of the *prothrombin time.* In this assay an effort is made to place prothrombin as the only limiting component of the coagulation system. Ordinarily this is done by restoring calcium ion and adding an excess of an active thromboplastin preparation to decalcified plasma (method of Quick). Supplementation of the fibrinogen level has also been recommended. The time required for a clot to form at 37.5°C. after adding calcium chloride is measured in Quick's test. Unfortunately the length of this time does not have a fixed normal value from laboratory to laboratory, so that the time required for normal plasmas to clot must also be known for that laboratory, and sometimes even for the particular lot of thromboplastin used.

Obviously the lower the prothrombin concentration, the longer the time requirement, but the relationship is not a perfectly inverse one. Figure 13.1 illustrates the character of the relationship. Ordinarily the prothrombin time is reported directly without attempting to obtain from it the corresponding concentration.

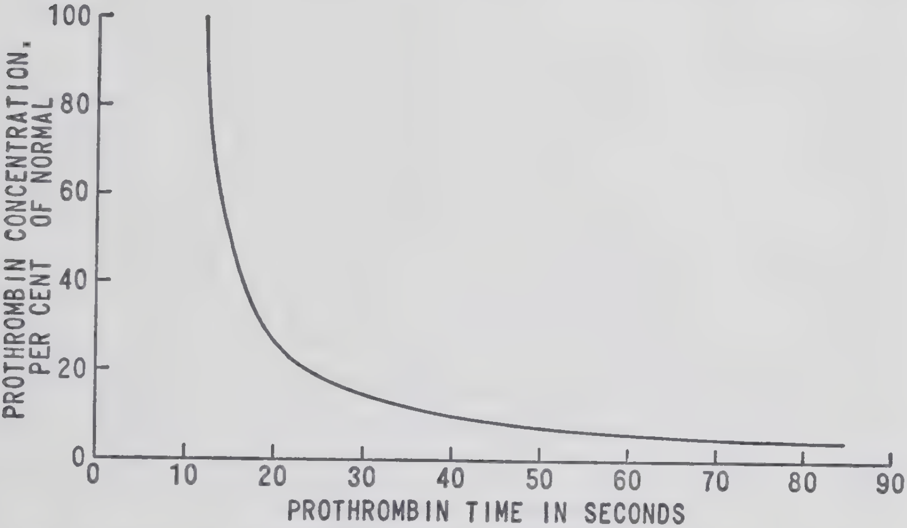


Fig. 13.1 Type of relationship existing between the prothrombin time and the prothrombin concentration. The curve is drawn from the relationship: percentage of normal prothrombin = 303/(prothrombin time − 8.7) (Quick), and taking the normal prothrombin time to be 12.0 sec. The position of the curve will be somewhat different for each specific set of laboratory conditions.

The action of thrombin is an exceedingly interesting one. It is a proteolytic enzyme that splits a polypeptide fragment (fibrinopeptide) from fibrinogen to convert the latter into *fibrin monomer.* This protein polymerizes spontaneously by hydrogen bonding to form long unstable fibrils. A *fibrin-stabilizing* factor in the presence of calcium ion gradually causes the formation of inter-unit S-S bridges to make the fibrin much more stable (Lorand; Scheraga and Laskowski).

Another interesting development is the discovery of the common amino acid sequence (*glycylaspartylserylglycyl*) in thrombin, trypsin, chymotrypsin, and phosphoglucomutase. The most significant amino acid in this sequence is serine, the hydroxyl group of which, under the co-catalytic action of a histidine residue in an adjoining chain, may receive an acyl or phosphate group transiently from the attached substrate. With certain abnormal substrates of the nerve-gas group, the binding may be more than transient (Dixon, et al.; Gladner).

In most enzyme assays the enzyme is permitted to work for a fixed period of time on a large *excess* of the substrate, under optimal conditions of *pH* and *temperature,* and in the presence of optimal concentrations of any cofactors that may stimulate the reaction. The reaction may be stopped sharply by adding a deproteinizing agent, and a characteristic product of the reaction then be measured. The time for reaction should be short enough so that negligible inactivation of the enzyme occurs during the incubation and so that there is neither an undue accumulation of the product nor depletion of the substrate. If the time is too short, the error in its measurement becomes significant.

Only if the above conditions are maintained does the extent of the reaction bear a direct relationship to the actual concentration of the enzyme. In developing a method, careful experimentation is used to find conditions under which the reaction appears to be of the "first order," i.e., the rate directly related to enzyme concentration. Even then, no attempt is made to convert the concentration back to the micrograms of enzyme present per unit of volume; instead, we report the number of *units of enzyme activity* found in a given volume. The unit may represent, for example, the milligrams of inorganic phosphorus released from β-glycerophosphate by the phosphatase in 100 ml. of serum in a given interval of time under a specified set of conditions. The unit then bears a label, usually of its author; for example, the Bodansky unit, the King-Armstrong unit, or the Russell unit. The complete designation reads, for example, X Russell units of amylase per 100 ml. of serum.

One should not assume that the time of the enzyme action can be doubled if the volume of the serum sample is halved or that the conditions can be manipulated in other ways. A careful laboratory provided with enough time verifies regularly that its methods give normal values for normal sera. Low values can readily be obtained if inhibiting impurities are present in the water or reagents or if the serum or blood is permitted to stand at room temperature too long. The instability of the prostatic acid phosphatase is notorious.

The problems of making sure that one is measuring the *amount* of an enzyme when he is assaying its *activity* become more severe when we analyze tissues, where access between substrate and enzyme may be modified by the method of preparation of the tissue. The proportion of the enzyme in an inactive form may also be modified in either direction.

Often the *specificity* of an enzyme assay is increased by various means. The pH can be adjusted to values unfavorable for related enzymes (for example, for the acid phosphatase and the alkaline phosphatase, respectively). This is not always completely successful. One should recognize that an extremely high *acid* phosphatase activity will lead to some increase of hydrolytic activity even at an *alkaline* pH. The specificity may be increased further by adding reagents that inhibit enzymes other than the one desired, or vice versa. For example, certain nonprostatic acid phosphatases are more strongly inhibited than the prostatic enzyme by alcohol or formaldehyde. When hemolysis occurs, a red-cell phosphatase that yields spurious results is released, but this can be controlled by formaldehyde addition. Tartrate addition is also used for increasing the selectivity of the assay.

Alkaline phosphatase assay presents a particular problem because of the ubiquity of enzymes of this character. Pathological increases arising either from liver or bone disease are usually what one seeks diagnostically. Although methods have been proposed for distinguishing activity from these two sources, reliance is usually placed upon other evidence concerning the patient in interpreting the results.

Why Do Enzyme Levels Change in the Plasma? Frequently the disease process causes an escape of the enzyme from the tissue rather than an increase in its production. Acute pancreatitis is an example. Similarly the prostate gland contains an enormous acid phosphatase activity; hence no increased production needs to be assumed in carcinoma. On the other hand the increased appearance of bone alkaline phosphatase in plasma is associated with increased osteoblastic activity, which presumably means more enzyme production.

With regard to the increase in the alkaline phosphatase in liver disease, one view holds that this is a *retention* phenomenon, i.e., a failure to excrete into the bile the normal amount of the bone phosphatase entering the plasma. This view is strengthened by the much greater tendency of the alkaline phosphatase to be increased in obstructive jaundice than in hepatitis, and by the higher level of the enzyme shown histochemically in the canniculae than in the hepatic parenchyma. We should note, however, that if this is a metabolic enzyme that must be eliminated by excretion, we have an apparently unique case. This is not the usual method for disposing of proteins. Another view is perhaps more plausible, namely, that the alkaline phosphatase appearing in the plasma in liver disease originates in the liver.

A nonspecific cholinesterase of the plasma is also believed to originate in the liver. The level of this enzyme, however, is decreased in liver disease. Either *excessive release* or *decreased formation* or *release* of an enzyme are plausible consequences of disease in an organ. The rationale of the changes of the plasma levels is not necessarily involved in their diagnostic utilization.

Transaminases specific to (1) the glutamate-to-pyruvate and (2) the glutamate-to-oxaloacetate transfers (in either direction) appear in the plasma when there is severe injury or necrosis of any of several tissues, liver and heart being of greatest diagnostic interest.

Diagnostic enzymology is a field still in its infancy. Even if we limit ourselves to easily sampled body fluids like the plasma, urine, and intestinal fluid, undoubtedly many of the instances where enzymes are discharged in abnormal quantities into these fluids remain to be discovered and exploited. But to restrict our interest to the enzymes that enter these fluids is absurd. As convenient a biopsy sample as the red blood cell now exposes the enzyme absence responsible for congenital galactosemia, certain drug-induced hemolytic anemias, and congenital methemoglobinemia. The leucocytes, representing perhaps the next most convenient biopsy sample, greatly increase the scope of exploration; bone marrow, still further. Of course many function tests exist and can be designed which are in fact, under appropriate conditions, *in situ* enzyme assays.

LIBRARY PROBLEMS

1. Set up and demonstrate the "sitting-drop" determination of total serum proteins.
2. Why is a pH as high as 8.6 or 8.8 chosen for electrophoresis of plasma proteins? Find and discuss an editorial by Youmans on the manner of expressing serum protein values.
3. In case 42332, *New England J. Med.*, **255,** 349 (1956), the serum electrophoretic pattern was described as normal, although there was a severe hypoproteinemia. Explain. Report what is meant by a cryoglobulin, a pyroglobulin, a macroglobulin. Is fasting plasma likely to contain more bound or free hexose?
4. Various formulas have been evolved for calculating the colloid osmotic pressure from the albumin and globulin levels, for example, $\pi = 45.2A + 18.8G$. If these are based upon the Howe analyses, would you expect them to be reliable? Comment on the precision with which an *edema level* for the serum proteins can be specified.
5. Can you suggest an unequivocal test for determining whether albumin is a principal form for the transfer of N to growing muscle, for example?
6. A remarkably clear discussion of the blood coagulation system is given by MacFarlane in Thompson and King, *Biochemical Disorders in Human Disease* (New York, 1957), pp. 122–51. In the light of this subject consider these two hypothetical propositions: (a) "One cannot predict, a priori, when he begins to investigate a biological phenomenon how complex it will prove." (b) "The very complexity of the present picture of the catalysis of the polymerization of fibrinogen proves that the picture is wrong." Why do the one-stage and two-stage methods for prothrombin give discordant results?
7. What changes in the serum protein pattern cause the Hanger *cephalin-flocculation test* to become positive in liver disease? What changes in the serum protein pattern cause the *thymol-turbidity test* of Maclagan to rise above four units in liver disease? What other turbidity and flocculation tests have been used in the past? Should all be continued?
8. Certain empirical tests on the cerebrospinal fluid, like the *Pandy test,* depend upon the protein concentration of that fluid. What concentration range is normal for these proteins and in what conditions is the level increased? Is retention of *both* the Pandy test and the cerebro-

spinal fluid protein determination desirable? Note results by both methods in case 44111, *New England J. Med.*, **258,** 546 (1958).

9. What do you conclude from reading as to the state of calcium metabolism in multiple myeloma?
10. Look up and report on the characteristic physical behavior of Bence-Jones protein. What curious feature of some of these was brought out by amino acid analysis?
11. Find and report a paper on the appearance of enzymes in the cerebrospinal fluid. Draw a schema to explain all plasma enzyme increases.
12. What enzyme assays of diagnostic value may be made on urine? On feces? Discuss these briefly.
13. Find the original paper that shows that a serum transaminase assay could be used diagnostically. Will hemolysis disturb the analytical results? Describe an assay procedure for serum transaminase activity and its interpretation. What expensive item of equipment does the original method use? Has a procedure been proposed which avoids this need? Cases that include transaminase assays are reported in *New England J. Med.*, **255,** 223, 234; **256,** 1060 (1957). In another case (*ibid.*, **258,** 1058 [1958]) a serum transaminase level of 2070 units was held to support hepatic necrosis rather than myocardial infarction. Why? Comment on the prothrombin finding in this case.
14. Find and report on a recent paper on the chemical nature of secondary *amyloid* deposits. What diagnostic test is often used to detect these? What difference is shown in the stainability of *primary* and *secondary* amyloid?
15. In case 44281, *New England J. Med.*, **259,** 88 (1958), a textbook statement is quoted to the effect that the congo red test for amyloidosis is apt to yield false positive tests when plasma protein is being lost into the urine. Trace the argument by which this is refuted. Discuss the various chemical data presented in this report. When the serum calcium fell to 4.8 mg. per 100 ml., could the serum contain any ionized calcium?
16. In case 44261, *New England J. Med.*, **258,** 1305 (1958), discuss carefully the two sets of albumin and globulin analyses presented. Would you anticipate the cephalin flocculation test to have become weaker or stronger in view of the changes in the plasma proteins? Discuss the other chemical observations.
17. Discuss one or more recent papers that report biochemical changes in schizophrenia.
18. Suggest an investigation (perhaps isotopic) to establish whether or not the liver clears extrahepatic alkaline phosphatase from the plasma to excrete it quantitatively into the bile.
19. Examine case 42352, *New England J. Med.*, **255,** 441 (1956). Is a

normal serum amylase level reported? Why may there be a higher amylase level in the abdominal fluid than in the serum?

20. In case 43041, *New England J. Med.*, **256,** 182 (1957), the protocol distinguishes between serum *total acid phosphatase* and *prostatic acid phosphatase*. How is such a discrimination made? What did the results obtained in this case indicate?
21. Find and report on a conference on blood enzymes. From the section on serum enzymes, discuss the directions that you feel diagnostic enzymology is likely to take in the future. In Chap. 1 an objection was raised to expressing diagnostic analysis as percentile scores, i.e., as the percentage of a population having an equal or lower serum level of a component. Do you think the objection applies equally to serum enzyme assays?
22. Find and discuss an essay by Luck on the problems of measuring the *pool size* and *turnover rate* for a given plasma protein.
23. Why have serum enzyme changes in disease been more promising diagnostically than changes in nonenzymatic proteins, for example, of glycoproteins or lipoproteins? Does this situation promise to change? Find and discuss a paper that illustrates why measures of nonenzymatic proteins are becoming more effective diagnostically. What is meant by DEAE-cellulose?
24. What plasma protein changes accelerate the rate of erythrocyte sedimentation? What diagnostic use is made of this rate measurement?

14

LIPID TRANSPORT

Absorption and Entry into the Circulation. The *physical nature* of the lipids introduces an inherently severe problem in their absorption and transport. In the intestine, the glycerol esters are extensively cleaved, but the fatty acids reappear mainly in triglyceride form in the intestinal lymphatics. The bile salts help to maintain lipids in suspension to facilitate their absorption. After years of uncertainty the lymphatics still appear to be the only important route of transfer to the blood stream of long-chain fatty acids, whether ingested in the unesterified or triglyceride form (Borgstrom). Interestingly, dietary linolenic acid may appear in the plasma of the rat in phospholipids and cholesterol esters rather than in glycerides (Mead).

The investigation of poor lipid absorption may be approached by the determination of fecal lipids (triglycerides and soaps) or by "tolerance tests" to see how much the ingestion of a test lipid (for example, vitamin A) increases the blood level of that lipid. The likelihood that fat-soluble vitamins are poorly absorbed in steatorrhea should be recalled. Radioiodinated triolein is also administered to investigate fat absorption.

Several hours after a fatty meal the plasma becomes milky from the load of fatty *chylomicrons* that are dumped in via the thoracic duct. These are microscopic droplets of triglyceride, which, however, migrate electrophoretically like proteins, so that they must be surrounded with protein. Essentially all the *increment* of triglyceride is in a form that will spontaneously rise to the top of the plasma. In a few more hours the milkiness disappears, and we might suppose that the chylomicrons have been removed by the liver or adipose tissues or by other tissues. The half-life of labeled injected chylomicrons in the dog is only 15 to 24 min. Clearly, however, a more complex sequence of events intervenes in their removal. Before proceeding with this subject, we must consider other forms of lipids present in the plasma.

The Lipoproteins. In the fasting individual most of the lipids are contained in microscopically invisible lipoprotein molecules, rather than as chylomicrons. Two very different types of lipoproteins have been isolated and studied, one migrating with the α_1-globulins and one with the β-globulins (Oncley). The α_1-lipoprotein is much the *denser* because its lipid content (12% cholesterol, 21% phospholipid) is much less than that of the β-lipoprotein (39% cholesterol esters, 9% cholesterol, 29% phospholipid, and, astonishingly, only 23% peptide chains!). Under ordinary conditions the latter *sediments* rather than *floating* upon centrifugation, but the NaCl concentration needs to be brought to only $1M$ to make it float. Its molecular weight lies in the neighborhood of 1,300,000. Significantly, fat-soluble vitamins and carotenoids are also detected in the preparation. The α_1-lipoprotein can be almost completely freed of lipid, apparently without denaturation or significant change of electrophoretic mobility (Scanu).

In addition to these two lipoproteins there is a spectrum of lipid-protein complexes migrating with the *α_2-globulins,* stretching to lower and lower densities, with the chylomicrons being the lightest. Some investigators who have approached the problem using the analytical ultracentrifuge, instead of recognizing the discrete classes of the α- and β-lipoproteins, have divided the lipid-protein complexes into arbitrary *flotation* classes (flotation = negative sedimentation; hence the designations $-S$ or S_f for these classes). The arbitrary ranges sometimes chosen unfortunately do not correspond with the more fundamental classification obtained by using multiple fractionation procedures. Table 14.1 is a summary of the lipoprotein fractions, setting forth equivalencies between the *electrophoretic* and *flotation* classifications.

Kunkel and Trautman have also correlated centrifugal and electrophoretic results as illustrated in Fig. 14.1. In this representa-

Table 14.1. Ultracentrifugally Separated Lipoprotein Classes. From Havel, *J. Clin. Invest.*, 36, 848 (1957).

FRACTION	NOMENCLATURE	DENSITY	ELECTROPHORETIC MOBILITY (STARCH)
I & II	Very low-density lipoproteins, including chylomicrons ($S_f > 10$)	<1.019	α_2-globulins
III	Low-density lipoproteins (S_f 0–10)	1.019–1.063	β_1-globulins
IV	High-density lipoproteins	1.063–1.21	α_1-globulins
V	Unesterified fatty acids + residual serum proteins	>1.21	albumin + all components

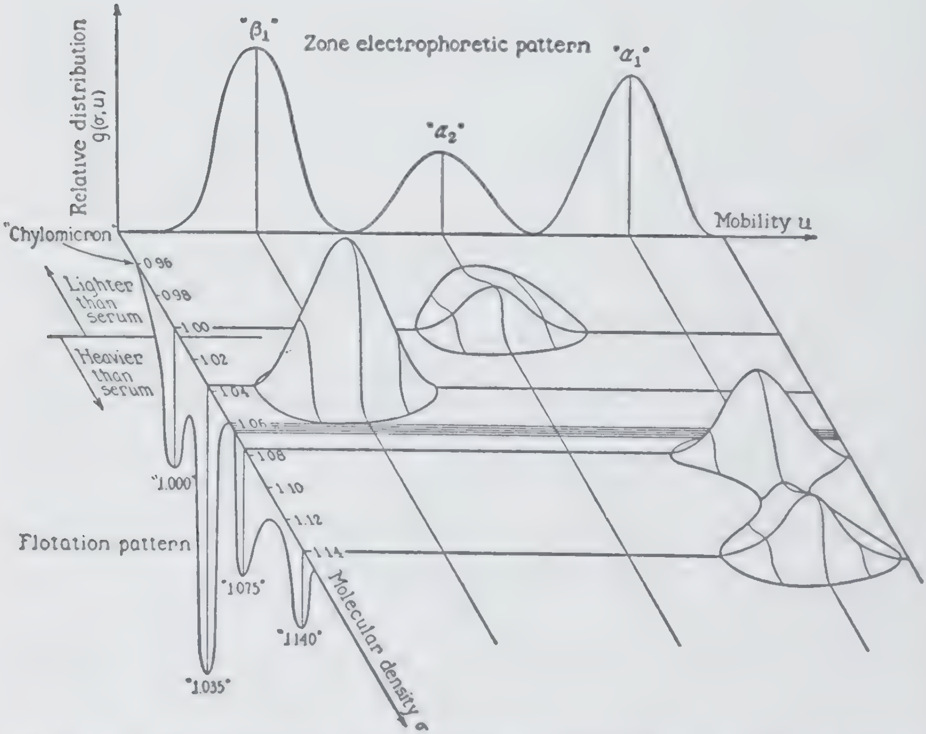


Fig. 14.1 Visualization of the classes of lipoproteins in plasma indicated by density determinations and electrophoresis. (Kunkel and Trautman, *J. Clin. Invest.*, 35, 641 [1956]).

tion the α_1-lipoproteins appear as the double-headed peak in the right foreground.

Are Chylomicrons Degraded in the Plasma? The polysaccharide heparin (and also certain other polysaccharides) upon injection causes the rapid clearance of lipemic plasma. If patients with idiopathic hyperlipemia and primary hypercholesterolemia are treated with heparin, the β-lipoprotein level decreases and α-lipoproteins increase. This is not a direct action, since heparin is ineffective when added to isolated plasma. Instead the heparin reacts with a tissue component to activate the real *clearing-factor,* which has been named *lipoprotein lipase.* This enzyme hydrolyzes the low-density and turbidity-causing complexes with the release of fatty acids, which are promptly bound by the serum albumin. In this form the fatty acids have a half-life of only 2 min. in the dog. These transient *albumin-bound unesterified fatty acids* apparently have a great importance in mobilization and transport of lipids from adipose depots and serve as an index of fat mobilization.

Information about the significance of the plasma unesterified fatty acids has been obtained in two ways:

1. By measuring the arterio-venous difference in plasma unesterified fatty acids;
2. By measuring the rate of disappearance of C^{14}-labeled palmitate introduced in the albumin-borne form (Dole).

Each method shows a shortcoming discussed in Chap. 1. In the first case the decision as to what flux has changed to cause an observed concentration change may be complex; in the second case we must be concerned as to whether the radioactive label may be lost by an idle exchange, without net flux, with a tissue pool.

Arterio-venous differences appear to indicate a lively addition of unesterified fatty acids to the plasma by fat depots and a lively removal by both skeletal and cardiac muscle (Gordon). The heart appears to remove enough fatty acid to account for a considerable fraction of its energy production. Both methods have shown that stimulated carbohydrate utilization inhibits the release of unesterified fatty acids from fat depots (Dole; Gordon), thereby revealing a mode of control of fat mobilization.

More recently, injection of the human pituitary growth hormone at levels required to produce growth has been found to raise the plasma unesterified fatty acids (Raben). Other hormone effects on mobilization of this fatty acid fraction have also been detected. An endocrine control of fat mobilization appears to be emerging.

Returning to the clearing reaction, apparently we have here an enzyme system for unloading transport lipids from lipoprotein complexes and for interconverting these complexes. Inherently the transport of water-insoluble lipids may be expected to require specific systems for loading and unloading the lipids from the protein carriers and possibly for interconverting transport forms. This is the first of such systems to be described.

Lipolytic attack on a low-density (high-lipid) lipoprotein should obviously lead to a second product besides the albumin-borne unesterified fatty acid; namely, a *higher*-density lipoprotein. Are the various plasma lipoproteins interconverted by a cycle of the gain and loss of lipid components? The α_1- and β-lipoproteins (peaks in right and left foreground of Fig. 14.1) are pre-eminently cholesterol- and phospholipid-containing lipoproteins. A ready exchange of cholesterol and phospholipid between these two classes has been shown (Gould), but this does not necessarily mean that the *polypeptide* portion has interchanged. In fact analyses for *constituent* and N-*terminal* amino acids of incompletely pure lipoproteins make it improbable that those with very high density can be transformed into those with low density. On the other hand these analyses are consistent with the possibility of interconversions *within* the wide spectrum of low-density α_2-lipoproteins (peak in middle background). Members of the latter group are also immunologically similar to each other. On the other hand characteristic changes in the concentration of the other two classes, as noted above, are seen during the clearing reaction. The nature of their participation is not yet clear.

Gould has shown with C^{14}-labeled cholesterol that rapid interchange of cholesterol occurs between the liver and plasma. The interchange is slower for other tissues. Cholesterol synthesis is fastest in the liver and the adrenal cortex, and in the ovary if corpora lutea are present.

Relation of Lipid Transport to Atherosclerosis. This disease is widely suspected of being a disease of lipid transport. Considering that the reactions of chylomicron degradation, as previously discussed, probably take place in plasma, it is perhaps not strange that the health of the vascular walls may depend upon their balanced progress. Gofman and his associates suggested that the incidence of atherosclerosis may be correlated with elevated levels of cholesterol-protein complexes in the flotation range S_f 12 to 20. This arbitrary range includes only a denser portion of the α_2-spectrum (peak in middle background, Fig. 14.1). Conflicting results and poor correlation have been reported by other investigators.

The β-lipoproteins, as separated by the Cohn and Oncley procedures, have been found by Barr, Eder, and Russ to be increased in atherosclerosis, whereas α_1-lipoproteins are decreased. These findings have been confirmed independently by paper electrophoresis. The β-lipoproteins were also increased in nephrosis and diabetes mellitus, diseases which predispose to atherosclerosis. The β-lipoproteins have been found to decrease after estrogen and to increase after androgen administration, and the high density α-lipoproteins have been found to change in the opposite direction. These findings may well be related to the much higher incidence of atherosclerosis in men than in women. Synthetic "metasteroids" are being investigated in which this effect of estrogens on lipoprotein distribution is exaggerated in relationship to other estrogenic actions.

This discussion of atherosclerosis will be restricted to the lipid transport aspect. The present state of knowledge does not yet appear to give a clear-cut, diagnostic value (in relation to atherosclerosis) to the measurement of lipids or lipoproteins in the plasma, but such developments may be anticipated. Simple analytical procedures for β-lipoproteins has been proposed by Bernfeld and by Antoniades. The flaw in the older diagnostic analysis for cholesterol, or even in the more refined differential analysis for cholesterol and its esters, lies in the failure of such analyses to inform us about the concentration of the various lipoproteins in which the cholesterol actually occurs and which appear to have quite varied metabolic significance. Total cholesterol analyses must accordingly have uncertain value in guiding therapy directed toward atherosclerosis.

Similarly measures directed at reducing cholesterol intake or absorption should take into account the well-established reciprocal relationship between cholesterol absorption and cholesterol synthesis. Accordingly a decreased exogenous supply simply stimulates endogenous production. Cholesterol feeding induces arterial plaque formation in rabbits, which have a highly labile cholesterol transport, but not in other species. More vulnerable reactions for which to design an inhibitor must lie in the sequence between coenzyme A and cholesterol (see Chap. 17). At the same time we should recall that an overproduction of cholesterol may not be the critical fault in atherosclerosis.

The Lipemia of Nephrosis. Hypercholesterolemia, frequently severe, is almost always observed in the nephrotic syndrome, along with increases of other lipids. The major increment is reported to lie in the β-lipoprotein fraction. This hyperlipemia has not been fully explained, but relevant experiments have been reported by Byers and his collaborators with an antibody-induced nephrosis in rats.

The absence of any changes in the rates of intestinal absorption, hepatic synthesis, or excretion of cholesterol was first shown. Therefore the source of the lipemia had to be sought elsewhere. The first plasma lipid change observed in experimental nephrosis, as the serum albumin level fell, was a rise of the triglyceride level. The injection of albumin caused a marked drop of the triglyceride level and also of the cholesterol and the phospholipid levels. The injection of heparin alone had little or no effect on the lipemia of either human or rat nephrosis. But albumin and heparin *together* decreased the lipemia of nephrotic rats even more strongly than albumin alone.

These investigators suggest that, because albumin serves as an acceptor for the fatty acids released by lipoprotein lipase, *intravascular lipolysis is blocked in albumin deficiency,* so that clearing cannot occur and the less dense lipoprotein complexes are not degraded. They propose that the extra cholesterol and phospholipid of nephrotic plasma accumulate secondarily because they are sequestered or *dissolved* by the excess plasma triglyceride. To support this view, they have shown that cholesterol is more readily extractable from nephrotic plasma than from normal plasma. Incidentally, cholesterol enters the blood from the intestinal lumen but not from the liver in an easily extractable form.

Heyman, et al., find, however, that the lipemia of experimental nephrosis appears *before* the hypoalbuminemia and therefore they question the proposed causal relationship.

The cholesterolemia of diabetes has been attributed to the elevation of acetylcoenzyme A levels resulting from the increased fatty acid breakdown, which by mass-action could be expected to stimulate cholesterol synthesis. Such an acceleration has been shown. The high plasma glyceride level in diabetes may arise from accelerated mobilization of fat. Solvent effects of lipids upon each other, as noted in experimental nephrosis, may complicate the picture. Here again the plasma level does not necessarily reflect the cholesterol synthesis rate.

Liver Disease. The most characteristic gross change in serum lipids in liver disease is an increase in the *free* cholesterol of the serum. Normally a remarkably constant portion of the serum cholesterol is found in the *un*esterified form, 26% to 30% by the Schoenheimer-Sperry method. This percentage frequently rises to 50% or 60% in cirrhosis or hepatitis primarily because of an increase in the free cholesterol level. With more severe damage the *esterified fraction may fall.* The more difficult differential determination of free and ester cholesterol becomes necessary for the results to be useful in liver disease. The technical difficulty of the fractional procedure should be taken into account in choosing diagnostic procedures for liver disease. Further specificity in determining the various lipoproteins and their free and total cholesterol content will probably be necessary before the rise in the free cholesterol can be understood. A beginning in such studies shows that the α-lipoprotein levels are *decreased,* leading to the view that the ability of the liver to synthesize high-density lipoproteins is handicapped in liver damage.

In obstructive jaundice the serum levels of phospholipid and esterified cholesterol also increase, in addition to the rise in free cholesterol. The cholesterol elevation has been called a *retention* phenomenon, but this is undoubtedly not an adequate explanation, since biliary excretion appears to play a minor role in the control of the plasma cholesterol level. The lipoproteins of very low density are especially elevated.

LIBRARY PROBLEMS

1. In case 43222, *New England J. Med.*, **256**, 1060 (1957), fecal analysis showed a high soap content. Does the suggested theory of a deficient secretion of pancreatic enzyme into the intestine seem the most plausible explanation?
2. On what two possible bases could a decrease in the plasma prothrombin develop in obstructive jaundice?
3. Find and report a recent summary concerning the possibility that a nutritional factor, possibly the quantity of highly unsaturated fatty acids in relationship to the quantity of saturated fatty acids in the diet is involved in the etiology of atherosclerosis.
4. Rinehart has reported that monkeys with a prolonged vitamin B_6 deficiency develop atherosclerosis, and Schroeder has produced evidence implicating pyridoxal deficiency and metal chelation in the etiology of atherosclerosis. Summarize this aspect briefly on the basis of two or three papers.
5. A few years ago textbooks suggested that the hypercholesterolemia of nephrosis served to help maintain the colloid-osmotic pressure of the plasma, the sterol compensating partially for the osmotic pressure of the lost protein. (a) Even if true, does this in any degree *explain* the development of the hypercholesterolemia? (b) Why is this hypothesis now unacceptable? (c) What procedures will show whether or not the cholesterol in nephrosis actually is dissolved in the plasma triglyceride?
6. In the Bloor method a colorimetric procedure (the Liebermann-Burchard reaction) is applied directly to a lipid extract of serum, whereas in the Schoenheimer-Sperry method the color reaction is applied only after saponification of the lipid extract. Investigate why the latter, longer procedure is to be preferred.
7. Find and report on one or more papers reporting intravenous human nutrition with emulsified fat. The injected fat globules rapidly become indistinguishable from chylomicrons and appear to be normally metabolized. Summarize the current status of this technique.

15

CIRCULATING CARBOHYDRATE

A Glucose-containing Environment for the Cells. Our blood contains glucose, not because we eat foods containing this sugar but because our metabolism is adjusted to form it from components of our food and to maintain an adequate glucose level as an important feature of the nutritive environment of our cells. Even after our liver glycogen is depleted by fasting, glucose continues to be formed from the breakdown of protein. Many of the more abundant amino acids (for example, glutamate, alanine, aspartate) are easily converted to glucose. Essentially as rapidly as glucose is removed from the blood by various tissues, it is fed into the circulation principally by the liver. Here again we must avoid the temptation to suppose, just because we find 200 mg. of glucose in 100 ml. of blood at 7:00 A.M. and the same level at 10:00 A.M., that this is the same sugar.

Just as in the case of amino acids, lipids, and inorganic ions, glucose distribution is not governed simply by its free diffusion into and out of freely permeable cells. Instead the transfer into cells appears to be chemically mediated, as though the glucose combined transiently with a membrane constituent during its passage. Such an arrangement may have the advantage of permitting *control* at the transfer step. In contrast to amino acid and many inorganic ions, glucose transfer is frequently not *concentrative.*

The Action of Insulin. Insulin *administration* increases glucose uptake by muscle and adipose tissue, stimulating not only glucose oxidation and glycogen storage but also protein synthesis in the former and fat synthesis in the latter. Insulin *deficiency* produces the reverse effects in these and other tissues, including liver. Most of these effects may be explained by a frequently shown stimulating action of insulin somewhere in the chain of reactions whereby extracellular glucose is introduced into cellular reactions. In cell-free homogenates only the final step in this chain is recognized, namely, the hexokinase reaction: Glucose + adenosinetriphosphate (ATP) → glucose-6-phosphate + ADP.

This reaction has been reported to be stimulated by insulin, but this effect has not been uniformly observed, and there are persistent indications that we should look to *an earlier step* so far detected only when the cells are intact, a step presumably concerned in sugar transfer into the cell.

First, sugars enter the cellular spaces more extensively (Levine) and more rapidly (Kipnis and Cori) if insulin is supplied, and a higher content of *free* glucose is found in muscle if insulin is administered (Park). Second, a competition among hexoses for passage through the chain is detected only when the cells are intact, pointing to a possible interaction of the sugars with a site of limited capacity prior to the entrance of the sugars into the hexokinase reaction. This prior step is believed to be a transport step and to be facilitated by insulin. The accumulation of α-aminoisobutyric acid by diaphragm is also stimulated by insulin (Kipnis and Noall). An alternative explanation, that insulin modifies the cell boundary to facilitate sugar diffusion, does not seem to explain readily the specificity of interaction among the sugars.

In evaluating this hypothesis of insulin action on sugar transport, a question has been raised as to whether the transport step is *rate-limiting* in cellular glucose utilization. If it is never rate-limiting, its acceleration may have no physiologic significance. In any event, until such a transport reaction is identified, the biochemical site of insulin action cannot be considered to be satisfactorily explained.

Possibly Significant Chemical Reactions of Insulin. Even when we know what reaction insulin is influencing, we need to discover *how* it influences this reaction. Some of the chemical reactivities of insulin may represent the specific property that causes this protein to modify the uptake of sugars and other substances; other reactivities may be "red herrings" with little consequence in the biological context. These properties present interesting possible distributional consequences.

1. *Reactivity with Zinc Ion.* Insulin crystallizes with the zinc ion occupying unidentified positions in the crystal structure. This metal is a component of many enzymes, but at 0.01 to 0.1 millimolar levels it strongly inhibits phosphoglucomutase. By forming a stronger complex with the zinc ion, insulin is able to relieve this inhibition. Quite another matter, however, is the question whether the zinc concentration ever gets high enough in the cell for this action to be significant.

2. *Reactivity with Proteins.* The complex that insulin forms with protamine is well known for its therapeutic use. Protamine is able to inhibit phosphorylase isolated from muscle, and here again insulin can remove the inhibition by out-competing with phosphorylase for the protamine.

Lipoproteins present in diabetic rat plasma bind insulin. These lipoproteins also can inhibit hexokinase and, as for protamine, insulin can relieve this inhibition. Special insulin-binding proteins may be present in the plasma of juvenile or insulin-resistant diabetics or in the plasma of insulin-treated patients.

Insulin is also able to react with hexokinase itself to stabilize this enzyme.

One can readily see from these examples the traps that are set for the investigator. Obviously not all the foregoing reactivities are responsible for the stimulation of glucose transport by insulin. Whereas the simplified cell-free system undoubtedly can provide the most decisive evidence as to the specific action of a hormone, the answer must always be checked for applicability at the levels of all significant components present *in vivo.*

When insulin comes into contact with muscle and other tissues, it becomes bound to the tissue. This reversible binding is probably related to the foregoing reactivities with proteins and is certainly a factor in the biological behavior of insulin. The insulin molecule is undoubtedly too large to diffuse into cells in any reasonable time; either it exerts its effect from the cell surface, or its entry must be *facilitated.*

Control of Insulin Supply. A rise in the blood glucose level produces an increase in the rate of insulin secretion, which in turn accelerates glucose transfer from the circulation. Insulin secretion can also be increased by neural stimulation and apparently also by administering certain sulfonylureas (for example, 1-butyl-3-*p*-tolylsulfonylurea). Agents of this class are under intense study for the control of the milder insulin-sensitive types of diabetes.

Various tissues have a high insulin-fixing ability, so that most of the hormone is removed in the first circulatory transit through the tissues. The isolated rat diaphragm has been used for studying this activity. When insulin labeled by iodination with I^{131} is administered, the isotope penetrates the cells and is concentrated in the cytoplasmic reticulum (microsomes), but this finding does not indicate that insulin has taken the same course. Furthermore the point of fixation of insulin could shift from the cell exterior to the microsome *after* the cell is broken. In fact we cannot even rely upon the I^{131} *in the plasma* to represent the insulin remaining there. Insulin is rapidly inactivated in the cells, partly by the proteolytic activity of *insulinase.* This rapid destruction is a characteristic and necessary feature of hormonal control, but one that does not simplify endocrine replacement therapy.

Other Hormones. Several other endocrine secretions are involved in the homeostasis of the blood sugar level. The release of *epinephrine* by the adrenal medulla in hypoglycemia is an important example. This hormone stimulates the formation of a cofactor necessary for the enzymatic conversion of hepatic dephosphophosphorylase to the active phosphorylase (Sutherland), presumably increasing hepatic glycogen breakdown and thereby producing a hyperglycemic effect. This conclusion implies that the quantity of the active enzyme normally present is a factor limiting glycogenolysis. Norepinephrine, a metabolic precursor of epinephrine and also an important hormone, does not share appreciably in this hyperglycemic action.

The secretory activity of the adrenal medulla is estimated by determining *catecholamines* in urine, a class to which epinephrine and norepinephrine both belong. Norepinephrine represents a major part of the normal urinary catecholamines, but much of this may arise from adrenergic nerves. Probably only minor quantities of the hormones secreted are actually found in the urine, judging from the low recoveries after their injection. Absorption chromatography permits separate analyses of the two catechols. The principal interest in the analyses comes in the diagnosis of pheochromocytoma and its distinction from essential hypertension. In the former disease the excretion is enormously increased, and the hormones may even be measured in the plasma.

Glucagon, first recognized as a contaminant of most insulin preparations, is a product of the α-cells of the pancreas. Like epinephrine it apparently accelerates glycogen breakdown by promoting phosphorylase activation. Differences in the action of glucagon and epinephrine are recognized but not yet explained. Although glucagon was only recently isolated, its polypeptide structure is already almost as well known as that of insulin.

A diabetogenic action of pituitary *growth hormone* appears to be inseparable from the growth-promoting action. A resistance to administered insulin is observed in acromegaly and in patients receiving this hormone. Accordingly the hypophysis is likely to be concerned in some types of insulin-resistant diabetes. Insulin-binding proteins in the plasma of treated diabetics contribute along with hormonal action to insulin resistance.

The adrenal cortical *glucocorticoids* stimulate gluconeogenesis and combat hypoglycemia. The basis of this action has not received unequivocal explanation. Changes in the output of these steroids seem to be absent or in the downward direction in human diabetes (excluding coma), and therefore such changes probably do not contribute to the accumulation of sugar in this disease (Conn and Fajans).

Thyroxine and triiodotyrosine accelerate the catabolism of carbohydrate. The increased catabolism of thyrotoxicosis contributes to the severity of the diabetic state.

The glucose tolerance test can serve to illustrate the homeostatic regulation of the blood sugar level. In the oral tolerance test a solution of 50 or 100 g. (or 1.75 g. per kilogram of ideal body weight) of glucose is given, and the blood sugar determined periodically for 3 hr. Glucose absorption is an active process the rate of which is not determined by diffusion (Cori). Certainly, extensive reactions (even with lactate formation) may occur during transit of the mucosa, but the *minimal* chemical reaction required for transport is not yet known. Abnormally flat tolerance curves are obtained in sprue. This variable, the intestinal absorption rate, can be eliminated by injecting the glucose intravenously, usually 0.5 g. per kilogram as a 50% solution in 4 min. or as a 20% solution over a 30-min. period.

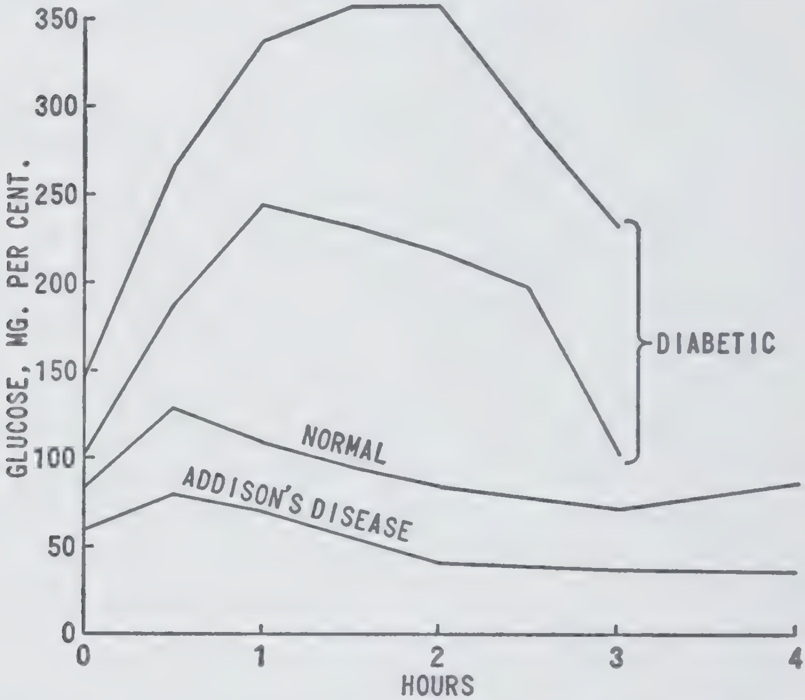


Fig. 15.1 Examples of normal and diabetic glucose tolerance curves and of the flat type of curve that may be obtained in Addison's disease. The upper curve is for a patient whose diabetes was already revealed by his fasting blood sugar level. The second curve is a case where the test was more necessary for the diagnosis. (From data of Dr. Stefan Fajans.)

The resultant elevated blood glucose level certainly accelerates directly the movement of glucose into cells, but a further increase results from the stimulated insulin output. This action is clear from the tendency of the blood sugar to pass briefly *below* the fasting level late in the tolerance test (Fig. 15.1) and also from a demonstrated rise in the apparent plasma insulin found on bioassay. The tissues of the diabetic patient, either secreting too little insulin or having its action antagonized by other agents, remove glucose from the blood stream more slowly than normally. Because a large part of glucose is taken up by the liver (with conversion to fat and glycogen), severe impairment of liver function can also slow the disposal of the extra glucose.

The glucose tolerance of a normal person is not precisely constant but depends instead upon his previous dietary history. In particular, a period of fasting or of subsisting on a carbohydrate-low diet leads to a notable decrease in tolerance.

The glucose tolerance reflects the summation of all the *accelerating* and *decelerating* influences that operate on sugar exit and entry into the circulation. Although the result may reveal a subnormal ability to deal with a heavy glucose load, it does not reveal whether the insulin deficiency is absolute or relative. The severity of human diabetes of neither the juvenile nor the adult type has been found to be well correlated with the extent of histological evidence for β-cell destruction in the pancreas. Furthermore, diabetic patients differ widely in sensitivity to insulin and their requirement for it, supporting a variable role of antagonistic factors and of absolute hypoinsulinism in the production of diabetes.

Flat or so-called *inverted* glucose tolerance curves (Fig. 15.1) are obtained in Addison's disease or in other types of hypoadrenalcortical activity. Concavity is, of course, a normal feature of the later part of glucose tolerance curves; therefore the word "inverted" is ambiguous in this connection. The removal of sugar from the blood is set into motion unbalanced by the opposed action of the glucocorticosteroids. A flat curve can also be obtained in malabsorption syndromes if the oral tolerance test is used. Incidentally, flat curves are not uncommon in healthy young persons.

The insulin tolerance test measures the degree of hypoglycemia produced by 0.1 unit of insulin per kilogram of body weight injected intravenously. A fall of the blood sugar to one-half or less of the fasting level in 20 to 30 min. (in the absence of hyperglycemia), followed by a rebound, is considered normal; a smaller fall represents insulin resistance.

Glucosuria frequently leads to the discovery of hyperglycemia but may also arise from defects in renal tubular transfer, which can be expressed as a decrease in the maximum rate of transfer, the T_{max} for glucose. Formerly, *thresholds* were used to describe this situation, but more detailed study has indicated that the limitation probably lies in the *amount* of a solute that can be reabsorbed per unit of time (actually 250 to 450 mg. of glucose per minute) rather than the plasma *level* that can be maintained by tubular transport. Accordingly, in some instances glycosuria arises from a chronically high glomerular filtration rate. The association of glucosuria with other tubular defects has been mentioned earlier. An unusual proportion of nondiabetic glycosuric patients later become diabetic.

Galactosemia is a hereditary disease in which a difficulty in converting galactose to glucose makes this sugar (and lactose, which contains it) hepatotoxic. Infants with this inborn biochemical lesion must be nourished on a lactose-free formula. The enzymatic defect has been shown to lie in the transfer of galactose from galactose-1-phosphate to form the specific nucleotide, *uridinediphosphogalactose* (Kalckar). Isomerization to glucose must occur in this nucleotide form, the uridine diphosphate "carrier" serving for a variety of such metabolic conversions. Conveniently this enzyme deficiency applies also to erythrocytes, so that a diagnostic biopsy sample is easily obtained.

Hypoglycemia. The tissues that seem most dependent upon an adequate level of glucose in their environment are cortical neurones. Evidences of large sympathetic and parasympathetic discharge and of the release of hormones of the adrenal medulla are evident in the symptoms of hypoglycemia. Irreparable damage occurs in the central nervous system if hypoglycemia is prolonged.

Hypoglycemia may arise from excessive insulin administration, from islet cell tumors, from glucocorticoid or growth hormone deficiency (hypopituitarism, some cases of primary adrenal cortical deficiency), and from defects in the deposition or breakdown of glycogen. The latter can result nonspecifically from diffuse liver disease or can result from one or several specific metabolic defects. Figure 15.2 illustrates the nature of the normal glycogen molecular structure, each ring representing a glucose unit. Most of the glucose units of glycogen are joined into chains by bonds from carbon 4 of the first sugar to carbon 1 of the second. At intervals, side branches occur by an additional linkage from carbon 6 to carbon 1.

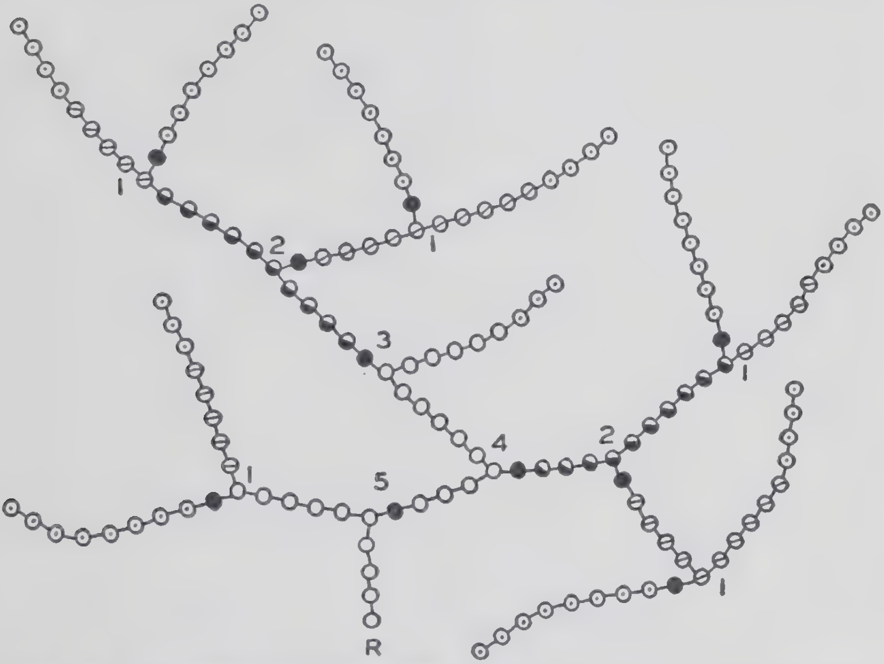


Fig. 15.2 The structure of glycogen. R is the reducing (aldehyde) end of the molecule. ⊙, ⊖ and ◒ are glucose units removed by the first, second, and third digestions with phosphorylase, provided that the glucose unit marked ● is split from 1,6 linkage by amylo-1,6-glucosidase between the first and second and between the second and third phosphorylase attack. (From Bentley Glass in a symposium on *Phosphorus Metabolism,* McElroy and Glass (eds.), p. 667, Baltimore, 1951. Derived from data of Larner and G. T. Cori.)

The side chains also branch, leading to the treelike structure illustrated.

As a consequence of this structure two enzymes are required for glycogen breakdown. Phosphorylase acts at the outermost 1,4 bond of each branch to release glucose-1-phosphate. When this action approaches a branching point by repeated cleavage of 1,4 bonds, it stops. The second enzyme (amylo-1,6-glucosidase, the *debranching* enzyme) must cleave the 1,6 bond to permit the breakdown to continue. In this case glucose rather than glucose phosphate is released.

Von Giercke's Disease. *Glycogen Disease with Normal Glycogen Structure.* The enzyme *glucose-6-phosphatase* is grossly deficient in the liver and kidneys in this disease, interrupting the release of glucose from the primary products of phosphorylase action. Chronic or intermittent hypoglycemia, hepatomegaly usually with glycogen accumulation, and interference with growth are characteristic.

Glycogen-Storage Disease with Abnormal Glycogen Structure. Formation of hepatic glycogen with *abnormally short outer chains,* arising from a demonstrated deficiency of the *debranching enzyme* (Illingworth, Cori, and Cori), or with *abnormally long outer chains,* conceivably arising from a deficiency of the *branching* (synthetic) *enzyme,* have been reported in two types of glycogen-storage disease. A rare deposition of abnormal glycogen in skeletal muscle may also arise from deficiency of debranching enzyme in that site.

Finally a cardiac glycogen-storage disease (with glycogen accumulating also in other sites) has as yet no known enzymatic basis.

Methodology. Most sugar analyses are based upon reducing properties, usually upon alkaline solutions of cupric ion protected from precipitation by chelating agents. A great variety of substances are able to reduce the Cu^{++} of such solutions, but fortunately, compared with glucose, most of these substances are not very abundant in body fluids.

In blood analyses, most of the nonsugar-reducing power is contributed by two sulfhydryl compounds of the red blood cells, glutathione and ergothionine. These may be avoided either by separating the serum for analysis or, more conveniently, by using deproteinizing agents that largely precipitate them along with the protein. Usually zinc sulfate and alkali are used together for this purpose to precipitate zinc proteinates and hydrous zinc oxide (the Somogyi procedure). An even more specific measure is now obtained quite easily by the use of the enzyme, glucose oxidase. The range for the *true* blood glucose is about 65 to 95 mg. per 100 ml., but if a method is used that includes the nonsugar-reducing substances, a range of

70 to 110 mg. per 100 ml. is more appropriate for the *apparent* blood sugar. This "double standard" frequently leads to the use of a normal range wide enough to cover all procedures, thereby sacrificing some of the acuity of the analysis. When blood sugar analyses are reported, the particular method used should be designated, especially if one of the less specific procedures is used.

One must remember that the ordinary blood sugar analysis includes such sugars as galactose or fructose as well as glucose when the former sugars are administered in tolerance tests. A specific galactose tolerance test requires a determination of this sugar per se. This is frequently approximated by destroying glucose first with baker's yeast before applying a reduction test.

Usually qualitative methods selected for *urine sugar* are not sensitive enough to yield positive results for the moderate amount of nonsugar-reducing substances present in normal urine. If, however, the urine is unusually concentrated, positive or equivocal (green) tests may be obtained for normal urines. A number of drugs also lead to excretory products with reducing activity, to cause false positive results. The use of paper strips infiltrated with glucose oxidase (for example, Testape or Clinistix) or of glucose oxidase in other forms now permits a simple and specific recognition of glucosuria.

The finding of a glycosuria not due to glucose demands that the urine sugar be identified. Paper chromatography is largely displacing a wealth of older methods for this purpose.

Sugar must be preserved from glycolysis in whole blood (for example, by the use of sodium fluoride) and from bacterial destruction in both blood and urine (for example, by thymol or toluene) if early analysis is not possible and especially if continuous refrigeration is impractical. Blood may be routinely collected in bottles containing sodium fluoride to serve both as preservative and anticoagulant.

LIBRARY PROBLEMS

1. Find and report one or more of the original accounts of the demonstration of stimulation of sugar *transport* by insulin.
2. Report a paper in which you believe that the conclusions are of

equivocal validity because of undue faith in the assumption that the fate of a protein can be determined by following the fate of I^{131} attached to it.

3. Does an increase of the concentration of a substance in the cell necessarily mean that all enzymatic reactions of the substance therein are accelerated? Explain.
4. Find an issue of a journal in which are gathered a number of papers investigating the mode of hypoglycemic action of the sulfonylureas. List the mechanisms of action proposed and indicate reasons for a preference among them. Do you receive a pessimistic or optimistic impression of the effectiveness of research when you consider the range of conclusions reached?
5. Attempt to discover whether a clear distinction had been established at the date of your writing in the actions of glucagon and epinephrine on phosphorylase activation.
6. Discover by reading and explain how the plasma inorganic phosphate level behaves during a glucose tolerance test.
7. In a case of malabsorption discussed in the *New England J. Med.*, **256,** 357 (1957), a pentose absorption test is used. Find the description of this test and discuss its use. Was the glucose tolerance normal in this case? Why was pancreatic disease considered unlikely?
8. Summarize the production of false positive urine sugar tests by medication.
9. Examine case 42231, *New England J. Med.*, **254,** 1090 (1956). Discuss the analyses of the tumor for epinephrine and norepinephrine. Discuss the glucose tolerance test reported. What is the significance of the urine analysis for "catechol"?
10. For case 41271, *New England J. Med.*, **253,** 28 (1955), graph and discuss the sugar tolerance and insulin tolerance tests made. Why should this patient show a low basal metabolic rate and a low serum protein-bound iodine? Is there a typographical error in the term *tri-iodothyramine* attributed to the discusser? Discuss the serum sodium level and the 17-ketosteroid and 11-oxysteroid excretion rates. What renal limitations do the Wilder test and related tests explore?
11. Discuss the purposes for which the following di- and polysaccharides have been injected: sucrose; inulin; dextrans; heparin.
12. Discuss the assay of plasma insulin on the basis of one or more research papers.
13. Discuss familial pentosuria on the basis of research papers.
14. Discuss on the basis of reading the carbohydrates involved in the blood-group substances.
15. Discuss a recent paper on the transport of glucose into erythrocytes.
16. Does a lowered pH of the extracellular fluid influence the glucose tolerance, i.e., the rate of glucose uptake by tissues?

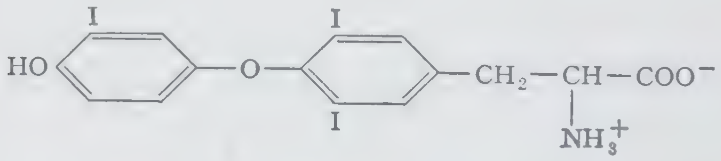
17. In case 44151, *New England J. Med.*, **258**, 749 (1958), is the recorded glucose tolerance abnormal? Discuss the origin of this change. Discuss also the low urine sodium and the serum alkaline phosphatase level. Note also a very low blood urea nitrogen level.
18. Why is cortisone sometimes given before the glucose tolerance test, and how does this influence the results?

16

IODINE AND THE CIRCULATING THYROID HORMONES

Many years passed after the discovery and identification of thyroxine in the thyroid gland before this substance was accepted as the *circulating* thyroid hormone. The long latency in its action on metabolism *in vitro* and a discrepancy between its activity and that of the whole thyroid substance led to doubt that thyroxine was the peripheral hormone. Finally in 1948 Taurog and Chaikoff showed in a series of quantitative tests that the hormone in blood behaved in every respect like thyroxine.

In 1952 a second circulating hormone, triiodothyronine, was discovered by Gross and Pitt-Rivers:



Subsequent studies have shown that triiodothyronine is present in much smaller quantities than thyroxine. The triiodo compound is, however, the more active physiologically and is also accepted as a circulating thyroid hormone. Part of it is formed by peripheral deiodination of thyroxine (Albright; Wilkinson & Maclagan). This reaction may be necessary for the hormone action of thyroxine. The conversion of the alanine side chain of both hormones to an acetic acid side chain has been observed in tissues; both products are nearly as active as their precursors.

Pharmacologically a wide range of related structures have a degree of biological activity. Other halogens or even nitro groups may substitute for the iodines; the hydroxyl group may instead be CH_3O-, and the alanine side chain may be replaced by a variety of chains that include amino or a carboxyl group or both. Attempts have been made to design analogs that could inhibit the peripheral effects of thyroxine. Butyl-3,5-diiodo-4-hydroxybenzoic acid interferes with the action of thyroxine, perhaps by interfering with its deiodination to triiodothyronine.

The precursors diiodo- and monoiodotyrosine are present in the thyroid gland in both the bound and free form. In goitrous cretinism substantial quantities of these two amino acids may enter the circulation; a lack of an enzyme deiodinating them may represent the genetic defect (Stanbury). Conceivably only the bound forms of the iodinated tyrosines are intermediates on the synthetic pathway, and a deiodination may be necessary to prevent an undue accumulation of the free iodinated tyrosines.

State of Thyroxine in the Plasma. The hormonal iodine is loosely bound to protein in the blood so that it cannot readily be separated by dialysis. When we precipitate the plasma proteins, the thyroxine, in contrast to the iodide ion, accompanies them. Therefore we have the somewhat misleading term *protein-bound* iodine, or PBI.

The behavior of the plasma hormonal iodine, as far as it is known, serves as an excellent illustration of the distributional considerations that underlie the efficient understanding of diagnostic analyses.

The first effort made to measure the *effective* concentration of the hormone was to separate the protein-bound iodine rather than to measure the total plasma iodine, thereby avoiding *one* ineffective form. But at this point unfortunately we have not yet reached the effective concentration (the so-called *chemical potential*) of thyroxine.

At normal levels the plasma thyroxine is largely bound to a specific α-globulin, designated as the *thyroxine-binding protein*, TBP (Gordon). This protein must be in equilibrium with a very low level of free thyroxine. Also this protein shows a definite saturation point in thyroxine binding. As more thyroxine is added to the plasma, first TBP becomes saturated and then albumin binds the further added thyroxine. Thyroxine is more firmly fixed than triiodothyronine to the binding protein and can displace the latter hormone from the bound form.

Now two possibilities will come to mind, either that the TBP-thyroxine complex or the *free thyroxine* in equilibrium with it represents the *effective* thyroxine; i.e., the form that tends to enter the cells to reach effector sites. Most of the evidence available concerning the mode of action of thyroxine supports the view that it uncouples oxidative reactions in the mitochondrion from the energy-trapping phosphorylations (Martius and Hess). Therefore this hormone almost certainly must enter the cell. At least for the main part of thyroxine transfer into cells, the TBP-thyroxine complex clearly does not serve; the more TBP one adds to a plasma sample, the *more slowly* the hormone leaves the plasma to enter various tissues equilibrated with plasma (Freinkel). In fact thyroxine already taken up from the plasma by tissues *can be withdrawn* by increasing the TBP level of the medium!

Furthermore the changes of the PBI level in disease frequently appear merely to be in response to modified TBP levels, i.e., to represent increases or decreases in the quantity of the hormone sequestered in the plasma in this complex. For example, in nephrosis or hepatic cirrhosis the fall in the PBI probably can be accounted for by a fall in the level of the specific carrier; the rise in the PBI in pregnancy, also without increased basal heat production, parallels the increase of the specific carrier level (Dowling, Freinkel & Ingbar).

Accordingly the bulk of the transfer of thyroxine from plasma to cells obviously occurs as free thyroxine. Thyroxine and triiodothyronine fit into the general cellular mechanism for concentrating amino acids (Hess) and may be transferred in the free form. We may be confident from the properties of thyroxine, however, that it becomes bound again by other protein systems in the cell.

It is still possible that, besides this bulk transfer, there may also occur a directed transfer of thyroxine to a specific acceptor, with the TBP-thyroxine serving as the donor. There is no evidence for such facilitated transfer, but no doubt the possibility will soon be checked.

In any case neither *the total iodine* nor the *protein-bound iodine* appears to represent the effective hormone level of the plasma. The PBI measure may frequently be merely a measure of the amount of thyroxine-binding protein present. A separate analysis of TBP may be necessary, or a measure of the degree of saturation of TBP (as for serum iron-binding capacity) should be useful to measure the "escaping tendency" of the plasma thyroxine. The problem is similar to that encountered with serum calcium except that only about 0.01% of the thyroxine may be in the unbound form!

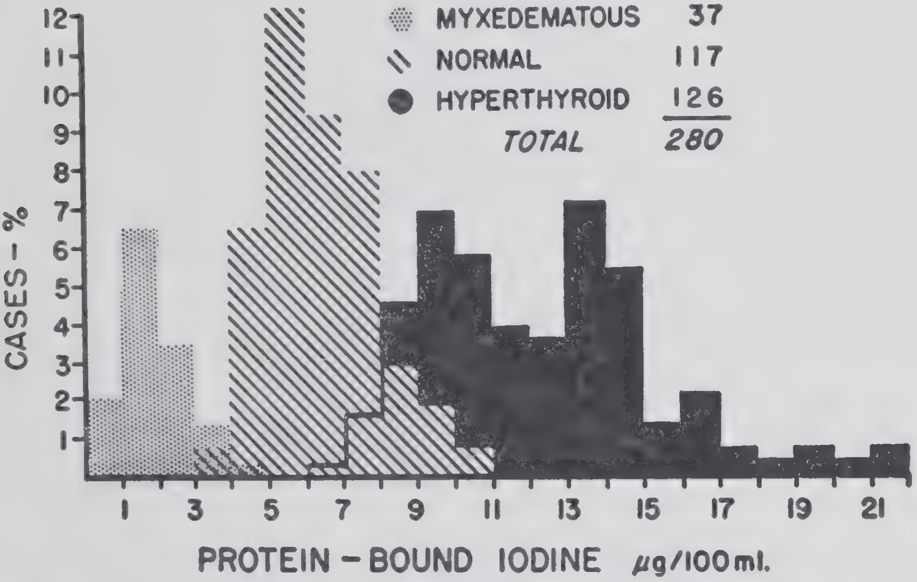


Fig. 16.1 Distribution of values for serum protein-bound iodine. Obliquely shaded areas, euthyroid patients; solid black, hyperthyroid; lightly dotted, myxedematous patients. (From Astwood, et al., in "Hormones in Blood," Ciba Foundation Colloquia on *Endocrinology,* vol. 11, London and Boston, 1957, p. 99.)

In spite of the large changes in PBI that can be caused simply by the changes in the carrier protein level, the PBI determination nevertheless has important diagnostic value. Figure 16.1 shows in the obliquely shaded area the values obtained in micrograms per 100 ml. of serum for 117 persons judged to be *euthyroid.* The solid black area shows the values for 126 patients convincingly diagnosed by other means as *hyperthyroid*, and the lightly dotted area shows the values for 37 *myxedematous* patients. The overlap is serious between euthyroid and hyperthyroid analyses. The generally accepted normal range of 4 to 8 μg. per 100 ml. could perhaps be revised to 4 to 8.5, judging from the illustrated results (Astwood); firm judgments clearly are not possible in the range 7 to 10 μg. per 100 ml.

Flux of Hormone from Plasma. Even when we do achieve a measure of the form of the hormone that is the effective one in entering cells, our result will still lack the dimension of *flux*, i.e., the rate of *renewal* and *removal* of hormone from the plasma. For example, triiodothyronine, although present at a much lower level, is moving much more rapidly than thyroxine through the plasma; here again we cannot safely decide about relative metabolic importance from plasma levels composed mostly of bound forms.

In *hyperthyroidism*, labeled thyroxine is much more rapidly removed from the plasma than normally, although the concentration of the thyroxine-binding protein does not seem to be changed. The "absolute organic iodine degradation rate" has been calculated as the number of micrograms of extrathyroidal organic iodine multiplied by its *turnover rate*. In normal persons values averaging 51 μg. per day were obtained; in myxedema, 18 μg; in thyrotoxicosis, 197 μg.; and in "hypermetabolism without endocrine disease," about 100 μg. per day (Sterling and Chodos). These findings introduce the possibility that overproduction of thyroxine could result from a "pull," caused by more rapid uptake of the hormone from the extracellular fluid, rather than a "push" from directly overstimulated glands. An adaptative increase of the tissue level of deiodinating enzymes (for example, of the kidneys) has been proposed as an explanation. A faster hormone uptake by the normal rat diaphragm from thyrotoxic plasma appears to prove, however, that the uptake-accelerating activity lies with the plasma rather than the cells (Hamolsky); perhaps an elevated *effective* level of plasma hormone will yet prove to be an adequate explanation.

The Analytical Method for Protein-bound Iodine. Current procedures obviate the *distillation* of iodine from an acid-digest of the serum proteins, and therefore have become less tedious, although still exacting. Instead the catalytic effect of iodine on the reduction of ceric ion by arsenite added to the digest is used to measure the iodine level. In this way the very tiny amounts of iodine (about a ten-millionth of a gram) present in perhaps a milliliter of serum may be measured. Contamination by mercury (which is also catalytic) or extraneous iodine is a serious hazard. Such a hazard is much more likely to arise from pharmaceutical preparations in the clinic (for example, diuretics, germicides, radiographic contrast media) than from laboratory preparations. In particular, iodinated contrast media for the biliary system may cause high PBI values for more than a year after their injection!

Flux of Iodide into the Thyroid. This flux has been extensively investigated for diagnostic purposes. About four-fifths of the body iodine is concentrated in the thyroid gland. Roughly 1% of this iodine appears not to be organically combined but present either as iodide or in a form in rapid equilibrium with iodide. The gland certainly has an iodine-concentrating activity, but before we can say that the gland has an *iodide-concentrating* mechanism, the proof must be completed that the apparent tissue iodide is actually free iodide. After blocking the iodination of tyrosine, the apparent concentration of iodide by the thyroid can produce a ratio of thyroidal I^- to plasma I^- of 25:1 in man and of 250:1 in the mouse.

As usual, at least two possibilities must be considered when a substance is accumulated in a particular site: Either the substance is bound or otherwise modified or restrained at that site, so that a real electrochemical concentration of the permeating form has not occurred; or the substance is *actively transported*. This question has not yet been answered for iodide and the thyroid gland, although a dependence upon oxidative phosphorylation (Freinkel & Ingbar) directs attention toward the hypothesis of active transport. Also the ability of the salivary glands to produce very high iodide levels *in the saliva* argues that equally high levels occur within the secreting cells. Perhaps it is not meaningful to speak of the active transport of iodide *as such*, since active transport presumably involves combination with a carrier; this means that active transport probably always occurs in a transiently modified form.

The thiocyanate ion interferes with iodide accumulation by the gland, whereas the hypophyseal thyrotrophic hormone powerfully intensifies the concentrating activity. The action of the thyrotrophic hormone has not yet been shown to be a direct one; a number of other changes are produced, including hyperplasia and an accelerated release of hormone by the thyroid; hence the sequence of the events caused by this hormone needs to be established. An artificially increased *thyroxine* level *diminishes* the plasma level of the thyrotrophic hormone, and thyroidectomy leads in a few days to an elevated thyrotrophin level. Hence thyrotrophic output is controlled by the effectiveness of its own action. The lower the iodine intake, the more vigorous is the thyroid uptake of a test dose of I^{131} (Stanbury).

The iodination reactions are not yet well enough understood to justify their review here. In fact the question remains unanswered as to whether iodide or elementary iodine or still another form actually reacts with the organic substrate. One of the reactions in this sequence is antagonized by the thiocarbonamide drugs; for example, thiouracil.

Diagnostic Measures of Radioactive Iodide "Uptake." The hyperactive thyroid removes a test dose of radioactive iodide from the plasma more rapidly than normally and also releases the hormonal iodine more rapidly. One can most simply observe this by measuring the urinary radioactive iodine; since the thyroid and the kidney compete for the administered iodine, whenever the gland takes up more, less is available for excretion. Thyrotoxic patients may be distinguished quite efficiently from euthyroid patients by this method.

One may instead observe an accelerated disappearance of total I^{131} from the plasma in an early stage, followed by an accelerated reappearance at a later stage. The plasma analyses become much more discriminating if the free iodine and the organic (protein-bound) iodine are determined separately. In this way the "conversion ratio" may be followed. Hyperthyroid patients show 45% or more of their total plasma I^{131} in the PBI fraction at 24 hr., whereas euthyroid persons show from 10 to 45% and hypothyroid patients less than 10%. These are empirical limits; the timing of the test is critical. Comparisons of the salivary iodine and the plasma PBI can accomplish similar discriminations, since the salivary iodine, although about 30 times more concentrated, varies more or less directly with the plasma *iodide* level.

Finally, the uptake rate may be investigated by the valuable method of direct scintillation-counting of gamma rays from the surface over the thyroid gland, after administering 1 to 5 microcuries of the I^{131}. This technique is especially valuable for localization studies, in which functioning or nonfunctioning nodules are mapped and distant functioning metastases sometimes found.

LIBRARY PROBLEMS

1. A recent paper reports that thyrotrophic hormone, labeled with S^{35} by conversion to a sulfate ester, upon injection into the chick causes more S^{35} to be localized in the thyroids than in other tissues. Do you think one will inevitably find that a hormone will be taken up more strongly by a target tissue than by other cells of the organism? Evaluate the method of labeling used.
2. Discover by reading how thyroglobulin was proved not present normally in significant concentration in the plasma; also the evidence for the escape of this protein into the circulation in Hashimoto's thyroiditis.
3. Outline a plan to discover either (a) what structural feature of the thyroxine-binding protein makes it a stronger thyroxine-binder than serum albumin and how the thyroxine is linked, or (b) whether various degrees of saturation of TBP with thyroxine are equivalent to various "escaping tendencies of thyroxine" from the circulation into cells, and whether a higher degree of saturation explains the more rapid thyroxine efflux from the plasma in thyrotoxicosis.
4. Discuss the concern expressed in case 41471, *New England J. Med.*, **253,** 926 (1955), that an intravenous pyelogram might have caused an artificially high serum PBI. Can you find a recent paper reporting the presence in blood of an unidentified organic iodine compound which subsequently was believed to have arisen from a gall-bladder contrast medium? Comment on the ability of such a compound to show such a long "biological life."
5. In case 43101, *New England J. Med.*, **256,** 465 (1957), discuss the chemical laboratory findings that are abnormal. Is triiodothyronine the usual form of thyroid medication? What does the pathologist mean in this case by "a cell failure at the biochemical level of hormone synthesis" in "histologically active thyroid tissue"?
6. Discuss the hazards in the use of radioactive iodine or the general problem of safe dosage in the use of radioactive isotopes.

7. We have encountered various agents that bind a blood constituent in which we are interested. Such a bound form may or may not be in the *miscible pool* of the constituent that we would measure by isotope-dilution methods. What determines whether the bound form is included or not? Is it the *stability* of the complex? Can you think of any highly stable complex that nevertheless rapidly exchanges its components with the environment? A stably bound hydrogen ion (for example, on $HPO_4^{=}$) may be considered. Can you think of any combination of low stability that nevertheless dissociates slowly and has little exchange?
8. Iodinated amino acids fall in a different order, when compared as to activity in uncoupling oxidative phosphorylation, than the order found for their activity in the intact animal. *In vitro*, *d*-thyroxine, *l*-thyroxine, and triiodothyronine have about the same activity. Does this raise serious doubt as to the validity of the uncoupling theory? Note also that both *d*- and *l*-ascorbic acid promote the *in vitro* enzymatic hydroxylation in tyrosine breakdown, but that only *l*-ascorbic acid serves in the intact organism. Similarly a large part of the ribonuclease molecule may be removed proteolytically without destroying its action, but this does not mean that the active fragment would serve in the intact organism. Suggest some explanations why structural requirements for a biologically active compound to be effective in the intact organism, must be more detailed than for it to be active in the test tube.
9. A determination of the serum *butanol-extractable iodine* has been proposed (Man, et al.). How can the "protein-bound" iodine be butanol-extractable? Compare the two methods as to significance. What advantage may the BEI determination have?
10. Recent results of Ingbar, et al., suggest that increasing the thyroxine-binding globulin content of plasma will cause extra thyroxine to move from the thyroid gland into the plasma. Could this serve to stimulate the metabolic rate? Or could TBG instead be useful for combating thyroid crisis by tying up plasma thyroxine?

17

STEROID HORMONE TRANSPORT

This chapter could discuss "Steroids and Transport," but perhaps this incompletely developed subject has received sufficient consideration in the foregoing chapters.

Transport is a key step for the hormones because these agents, by definition, must be transported through the blood and interstitial fluid from a site of synthesis to one or more sites of action. Until recently, however, measures of the circulating levels of the hormones have been obtained rather rarely because of the absence or technical difficulty of the methods. With the emergence of a growing set of blood assays, the consideration of hormone transport appears to represent a fruitful approach to endocrinology. Knowledge of the transport of some of the other hormone types, such as the *peptide* or *protein* hormones (except for insulin), is perhaps less developed than for steroid transport; hence the latter group is selected for discussion.

Types of Steroids To Be Considered. The structural similarities among the classes of steroid hormones are not so great as appears superficially to be the case. One great difference lies in the configuration of rings *A* and *B* (Fig. 17.1). These are aliphatic in character, except in the estrogens, which means that the hexagons are *not planar* as we ordinarily draw them. Instead the upper and lower carbon atoms must lie either in front or behind a plane formed by the remaining four carbon atoms of each ring. Investigators now believe that one of these carbon atoms lies in front and the other behind the planes in rings *A*, *B*, and *C*.

The two isomeric configurations of rings *A* and *B* may be differentiated most simply by considering the relative positions of the angular methyl group at C_{10} and the angular H at C_5, rather than the configurations of the rings themselves. As a convention we always place the structure with the angular methyl group at C_{10} toward

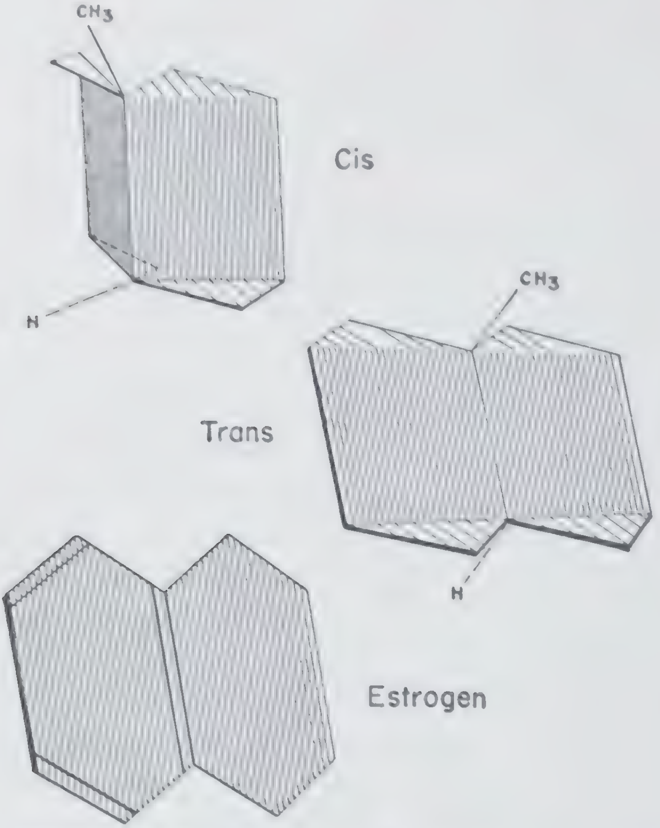


Fig. 17.1 Showing the *cis-* and *trans*-configurations of the angular groups of rings *A* and *B* of the steroids. The *adrenal* and *progestational* steroids are based upon the *cis*-configuration, whereas androstane, parent hydrocarbon to the *androgens*, has the *trans*-configuration. In the estrogens ring *A* (and probably ring *B* also) is rendered planar by the aromatic system of double bonds, so that such *cis-trans* isomerism is excluded. (The configuration that the ring assumes may be ignored as long as the steric relationships among the attached groups are kept in mind.)

the observer. If the angular H at C_5 also protrudes toward the observer the structure is known as the *cis* or *normal* configuration, which is characteristic of the adrenal and progestational steroids. If instead the C_{10} angular methyl group protrudes forward and the C_5 angular hydrogen backward (Fig. 17.1), we have the characteristic *androgen* structure and a derivative of the parent hydrocarbon *androstane.*

A great many of the active hormones, however, have a double bond between carbons 4 and 5 (as illustrated in Fig. 17.2). The alert student will protest that now there will be no angular hydrogen at C_5 and the configurational distinction between the androstane and pregnane series will disappear for these active hormones. Figure 17.1 presents the current conception of the *cis* and *trans* configuration. You will see that this picture preserves the distinction between the isomers even when the hydrogen at C_5 is missing.

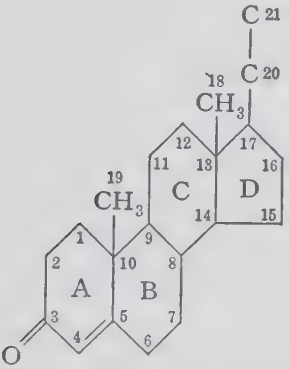


Fig. 17.2 Numbering of steroid structure.

Variation can also occur as to the orientation of the C_3-hydroxy group (except in estrogens) and the C_{17}-hydroxy group. For reference the molecule conventionally is oriented again with the C_{10} angular methyl group toward the observer; these hydroxyl groups are then said to have the *alpha* configuration when they point *away* from the observer, and the *beta* configuration when they point toward him; a dotted-line bond is used to indicate the α(away)-orientation.

If a hormone has any side chain at C_{17}, the chain is 2 carbons long, having a methylketone structure in progesterone and a hydroxymethylketone structure in the adrenal corticoids, a feature that produces some valuable analytical reactions. The latter hormones also have an oxygen-containing group at C_{11}, except for *deoxycorticosterone* and *11-deoxyhydrocortisone,* and may have a hydroxyl group also at C_{17}. The newly discovered hormone *aldosterone* contains an aldehyde group at C_{18} and is believed to exist also in a second form, the *hemiacetal* illustrated in Fig. 17.3. Although many steroids have been prepared from the adrenal cortex, the only ones believed to be secreted in significant quantity by the human gland are hydrocortisone, corticosterone, aldosterone, several androgens, and possibly an estrogen.

Biosynthesis. Steroids are synthesized from the acetyl radicals of acetyl-CoA. Figure 17.4 summarizes briefly some of the proposed earlier steps, while Fig. 17.5 below presents some of later stages. The perfusion of isolated beef adrenals (Pincus) has shown that the gland liberates 7 to 30 times its own content of various steroids in an hour. Introduction of ACTH into the perfusion fluid led to a sixfold increase in steroid release in a single passage. Clearly the gland does not store much hormone, the synthetic rate and the release rate being very similar. Apparently the gland responds to stimulation by accelerating the *synthetic* rate. Although corticosterone is most abundant in the beef gland, this hormone is not the one secreted most rapidly *in vitro*. ACTH accelerates synthesis from cholesterol, probably at one of the early stages because synthesis from progesterone is not stimulated (Hechter). Interestingly aldosterone output is controlled only to a minor extent by pituitary ACTH, but possibly instead by a hormonal factor originating from the hypothalamus (Farrell). The variables that modify aldosterone output appear to include the blood volume.

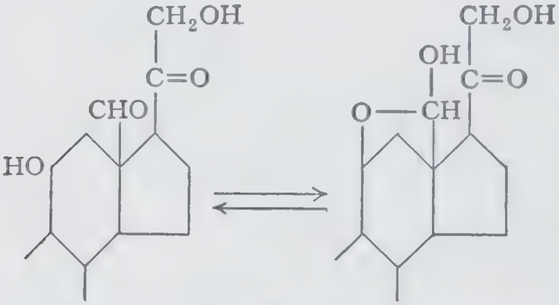


Fig. 17.3 Forms of aldosterone. (Rings *C* and *D* only shown.)

Metabolic Modification. Investigators have long suspected that hormones act not merely by their *presence* in the cell environment

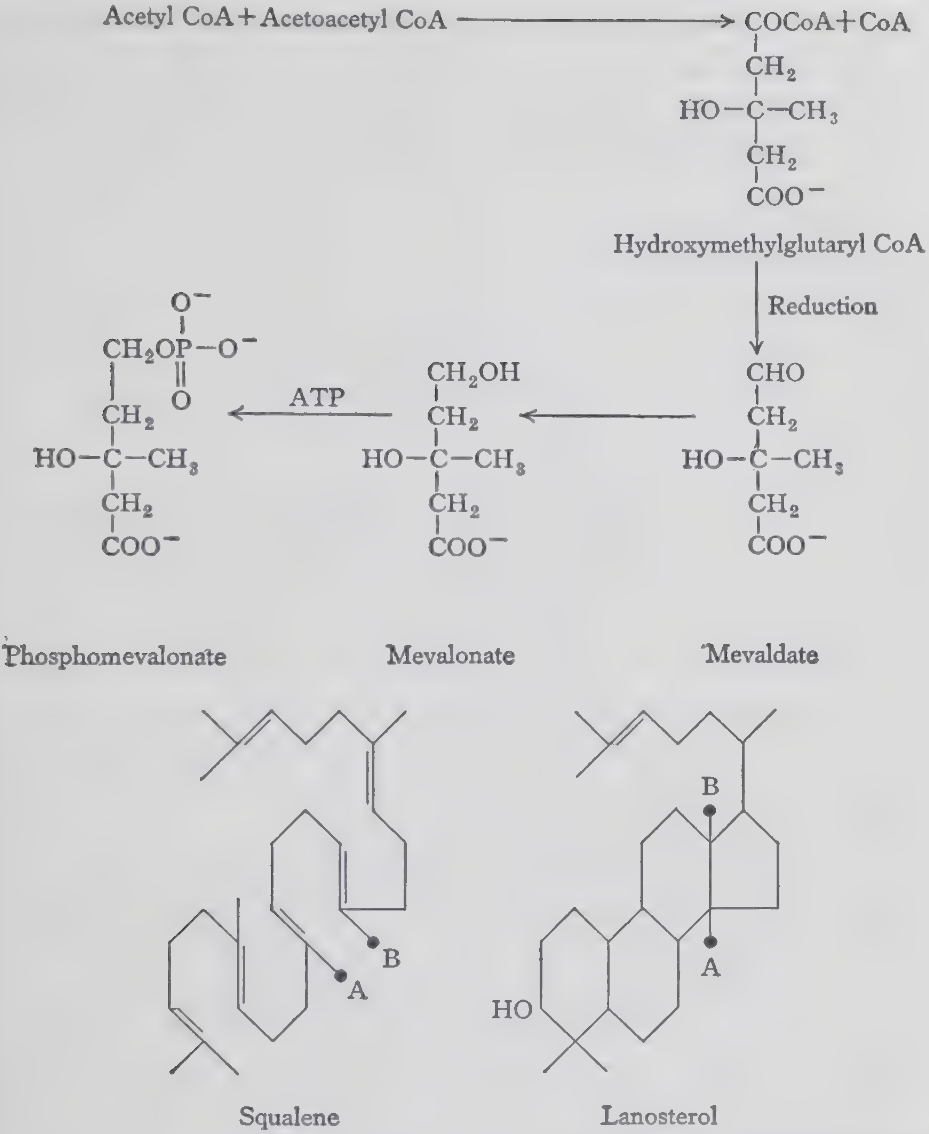


Fig. 17.4 Proposed biosynthetic pathway to sterols. Phosphomevalonic acid, upon splitting off phosphate, H_2O, and CO_2, can yield isoprene; hence it is equivalent to isoprene in level of oxidation. Six phosphomevalonate molecules join together to form the long *squalene* molecule; in every case the branch in the isoprene chain is *distal* and the configuration at the double bond is *trans*. Closure to a ring system yields lanosterol. This then loses the *A* methyl group to form 14-norlanosterol and is then oxidized to the corresponding 3-keto compound. Other details such as mode of removal of the two methyl groups at C_4 are not yet known. The step at which hydroxymethylglutaryl CoA is reduced appears to be particularly accessible to regulation. (Ciba Foundation symposium, *Biosynthesis of Terpenes and Sterols,* publication anticipated in 1959.)

but by *entering into chemical reactions* and therefore by being converted to something else. Therefore the numerous modifications that steroid hormones undergo in the body receive great interest. Various chemical groups may be either oxidized or reduced; in addition the steroids may be extensively conjugated with glucuronate, with sulfate, and probably with still other substances (Bush). The latter conjugation reactions may serve to inactivate the hormones and frequently to accelerate excretion by the urinary route.

A characteristic metabolic modification of administered corticosteroids is a reduction of one or two keto groups (the C_3 and/or the C_{20} keto group) to form "tetrahydro" derivatives, the *cortols* and *cortolones* (see pp. 217, 218).

Recently the oxidation or dehydrogenation of 17-β hydroxyl groups has been very significantly implicated in metabolic regulation by steroids. This development was initiated by the discovery by Villee that certain estrogens stimulate the reduction of diphosphopyridine nucleotide (DPN) in placental extracts when isocitrate is added. The accelerated reaction turns out to be a transfer of hydrogen between TPNH (reduced triphosphopyridine nucleotide) and DPN (a *transhydrogenase* action) and not to be concerned directly with isocitrate (Talalay).

The two pyridine nucleotides, DPN and TPN, serve entirely different metabolic functions because most dehydrogenase systems can receive hydrogen from one of these but not the other. TPNH can act as a reducing agent in a number of synthetic reactions that take place outside of the mitochondria and in which DPNH cannot participate. In various mammalian tissues the TPN is mainly in the reduced (TPNH) form, whereas more of the DPN usually is the oxidized form. Modification of these proportions, which can be accomplished by the transhydrogenase reaction, $\mathrm{TPNH} + \mathrm{DP}\overset{+}{\mathrm{N}} \rightleftharpoons \mathrm{DPNH} + \mathrm{TP}\overset{+}{\mathrm{N}}$ can modify in the cell the nice balance between synthesis and degradation required for growth and function.

Because the reversible dehydrogenation reaction between estradiol-17-β and estrone, for example, can exchange hydrogen with *either the DPN or TPN system, the steroid dehydrogenase system acts as a transhydrogenase* that can modify the critical balance of cellular synthetic and degradative reactions (Talalay). Other 17-β hydroxysteroids act analogously with other enzymes to serve as transhydrogenases. The affinity of the enzymes for the steroids is so high that activity is obtained at the very low physiological levels of the steroids.

If the foregoing interpretation of regulation by steroids through transhydrogenase action is fully verified, it will represent a classic exposition of endocrinologic effects.

This behavior seems likely to explain many of the actions of the estrogens and the androgens; perhaps analogous reactions of the C_3, C_{11}, or C_{20} oxygen groups may extend the concept to the other steroid groups. Differences in steroid affinities and specificities of the enzymes in various tissues may explain the differential loci of action; for example, of estrogens and androgens.

Urinalysis for Steroids. Even with the advantages of C^{14} labeling, the various steroid hormones are by no means recovered quantitatively as excretion products in the urine after administration; nevertheless the formation of characteristic excretory forms permit diagnostic estimates of the rates of production of particular hormones. Analyses of 24-hr. urines for such products so far have been diagnostically much more valuable than blood steroid analyses.

Urinary estrogens. The excretion of estrogens varies cyclicly during the menstrual cycle (best shown by chromatographic separation and separate analyses for estradiol, estriol, and estrone) and is enormously increased during pregnancy. In toxemic pregnancies the excretion rate is considerably smaller than in normal pregnancies. Biological methods remain important in routine estrogen assay.

Pregnandiol (pregnane-3α,20α-diol) *glucuronide* is the chief urinary metabolite of progesterone. Its excretion may be measured to determine the adequacy of progestational secretion by the placenta during pregnancy, to guide substitution therapy.

The *17-ketosteroid* excretion rate represents a crude summation of the production of gonadal androgens and of certain hormones of the adrenal cortex. The 17-ketosteroids arising from the adrenal cortex are mainly metabolic products, both of C_{19} and C_{21} steroids. The basic reaction is a color production with *m*-dinitrobenzene, the

Zimmermann reaction. Estrone is also a 17-ketosteroid, but in the ketosteroid method the acidic estrogens are ordinarily eliminated by an alkaline wash of the steroid extract. The average excretion rate in men has been recorded as 14 mg. per day with a range of 8 to 20 mg. per day; the range for women is roughly 5 to 15 mg. per day. In Addison's disease male patients may excrete 2 to 4 mg. per day; women, understandably, less than 0.5 mg. per day. Excretion rates as high as 10 mg. (men) or 3 mg. (women) are, however, not uncommon.

The effects on steroid excretion of hyperadrenocorticalism of the following classes will be considered:

1. *Cushing's Syndrome:* (a) caused by tumor; (b) associated with bilateral hyperplasia
2. *Adrenal Virilism or Adrenogenital Syndrome:* (a) caused by tumor; (b) associated with hyperplasia (congenital adrenal hyperplasia)

In Cushing's syndrome the *glucocorticoid* secretion, by definition, is excessive; in the latter two situations the *androgenic secretion* is excessive. Hence the 17-ketosteroid output is more strongly and uniformly increased in the adrenogenital syndrome and only irregularly in Cushing's syndrome. Particularly in virilism due to tumor, very large amounts of ketosteroid (largely dehydroisoandrosterone) are excreted.

Steroid suppression tests are applied to distinguish between tumors and hyperplasia as sources of hyperadrenocorticalism. A test dose of up to 200 mg. of cortisone, or 10 mg. of 9α-fluorohydrocortisone, or 50 mg. of prednisolone per day is considered to cause a fall in the ketosteroid secretion in hyperplasia but not in the presence of tumor. The distinction is recognized under present techniques to be rather unreliable in differentiating the two forms of Cushing's syndrome, but more reliable in differentiating the virilizing tumors on the one hand, from virilism associated with adrenal hyperplasia on the other.

Congenital adrenal hyperplasia appears to represent an inborn error in the hydroxylation of progesterone to hydrocortisone in the adrenal cortex so that C_{19} intermediates and their metabolites accumulate (Fig. 17.5). Accordingly the ketosteroid excretion is increased and glucocorticoids excretion decreased. The depressed level of plasma hydrocortisone removes the usual inhibition of ACTH release (this being the mode of control of corticotrophin output), leading to ACTH overproduction and to a consequent further formation of the androgenic metabolites.

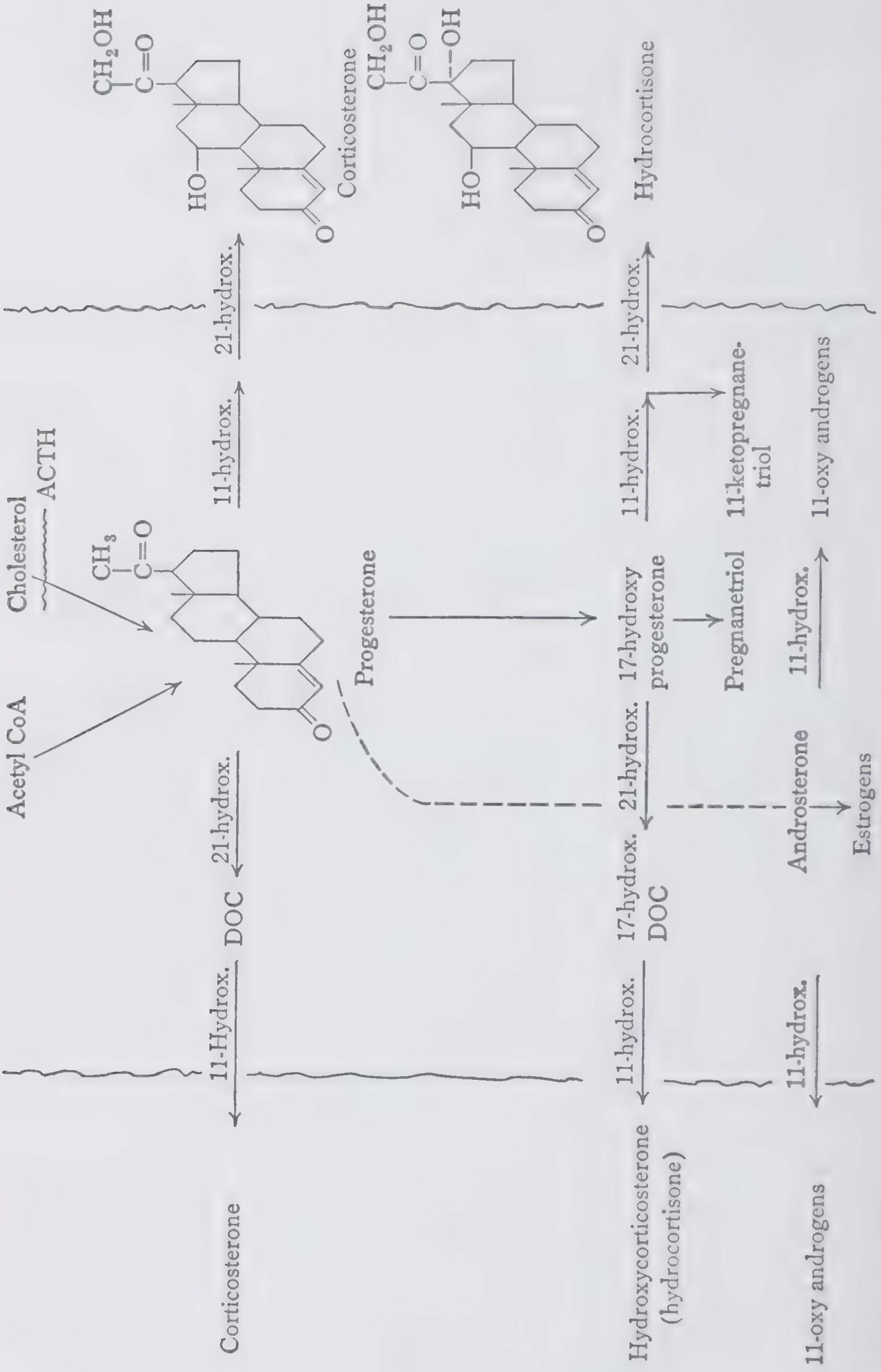
On this basis the result of the steroid suppression test becomes understandable. When cortisone is supplied, the ACTH overproduction is again suppressed, thereby decreasing the androgen accumulation and ketosteroid excretion.

The scope of enzymatic defects in steroid biogenesis is not yet entirely clarified, although the importance of these sources of endocrine disease is clear.

Efforts to improve the rather unspecific ketosteroid method are taking the direction in line with modern trends of improving separation of steroids of different significance from each other, especially by column chromatography, rather than of improving the color reaction itself.

Urinary Corticosteroids. The ketosteroid method suffers both from low specificity and from the dual origin of reactive steroids: adrenal cortical and gonadal. Methods which are based upon the sugarlike side chain of the cortical steroids (Fig. 17.6) are more satisfactory than the 17-ketosteroid analysis for investigating adrenal cortical function. Early methods were based upon reducing methods, not unlike those for sugars. *Triphenyltetrazolium* has proved to be a particularly useful oxidizing agent (Mader and Buck). The reaction is given whether or not 11- or 17-oxygen-containing groups are present, so that corticosterone and deoxycorticosterone react, as well as the 17-hydroxy corticosteroids, like hydrocortisone.

A method with a narrower specificity, requiring the presence of the 17-hydroxyl group (i.e., a complete "dihydroxyacetone" residue), is the *Porter and Silber method.* This is based upon the production of a yellow color with phenylhydrazine and sulfuric acid. The sample is treated with β-glucuronidase to cleave glucuronides; this step is followed by chloroform extraction of the steroids before colorimetry


Acetyl CoA
Cholesterol
ACTH
CH_3
$C=O$
O
Progesterone
21-hydrox.
DOC
11-Hydrox.
Corticosterone
11-hydrox.
21-hydrox.
HO
CH_2OH
$C=O$
O
Corticosterone
17-hydroxy progesterone
Pregnanetriol
11-hydrox.
11-oxy androgens
11-hydrox.
11-ketopregnanetriol
21-hydrox.
HO
CH_2OH
$C=O$
OH
O
Hydrocortisone
21-hydrox.
Androsterone
Estrogens
17-hydrox.
DOC
11-hydrox.
11-hydrox.
Hydroxycorticosterone (hydrocortisone)
11-oxy androgens

(Glenn and Nelson). About 4 to 10 mg. have been found excreted by normal men and women.

A third corticosteroid method, the so-called *17-ketogenic method* of Norymberski has the advantage of including a group of 17-hydroxycorticosteroid excretory products in which the 20-keto group has been reduced to a hydroxyl group (*cortols* and *cortolones*). These compounds, as well as their 17-hydroxy corticosteroid precursors, have their entire side chain split off by an action with sodium bismuthate to form 17-ketosteroids, which are then measured by the Zimmermann reaction.

Each of these three methods is applied to the lipids extracted by an organic solvent after mild hydrolysis, preferably by β-glucuronidase. The excretion rate found by each method is increased in Cushing's syndrome and decreased in Addison's disease. Perhaps none of the procedures gives the precise secretion rate of any single class of steroids, but each mirrors such rates. For example, only about one-quarter of a test dose of labeled hydrocortisone was recovered in urine as phenylhydrazine-reactive material (Peterson and Wyngaarden). The bismuthate method should give a better recovery, since the cortols and cortolones arising from hydrocortisone metabolism are 17-ketogenic.

When Cushing's syndrome is associated with bilateral adrenal hyperplasia, an abnormal response in corticosteroid secretion tends to result from ACTH administration; but this is not present in Cushing's disease due to tumor. The response in corticosteroid ex-

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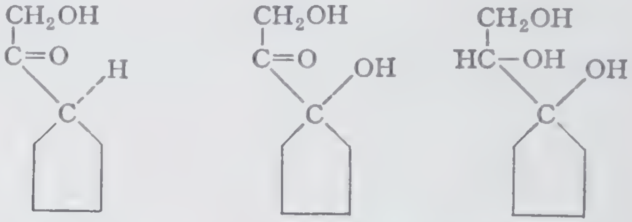
Fig. 17.5 Possible role of two different hydroxylation defects in virilizing adrenal hyperplasia. Hydrox. = hydroxylation. The presumed consequences of deficient 11-β-hydroxylation are shown to the left; those of deficient 21-hydroxylation, to the right. In either case corticosterone and hydroxycorticosterone formation is only partially blocked, stimulated ACTH formation partially compensating for the defect. In both defects androsterone and pregnanetriol are formed in abnormal quantities. If the 21-hydroxylation is defective, the 11-oxy derivatives of these two substances can be expected also to be characteristically overproduced. If the 11-hydroxylation is at fault, deoxycorticosterone and its 17-hydroxy derivative should form (thereby perhaps accounting for the observed hypertension). In this case the 11-oxy androgens and pregnanetriol should not increase. A salt-losing property of some of these patients is not accounted for. (Adapted from Wilkins, *The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence,* 2nd ed., Springfield, Ill., 1957, pp. 333–35.)

cretion upon ACTH administration is also a valuable measure of the *adrenocortical reserve* in suspected hypocorticalism.

Of the foregoing urinary analyses, the 17-ketosteroid method is generally available in the clinical laboratory, and one or another of the methods for the corticosteroids is usually available in most large hospitals at the present time.



	Hydroxymethyl Ketone Structure of Corticosterones	"Dihydroxy-acetone" Structure of 17-Hydroxy Corticosterones	Side Chain of Cortisone Metabolites, Cortolones Cortols
Reducing properties (e.g., for tetrazoliums)	Yes	Yes	No
Porter-Silber test (phenylhydrazine)	No	Yes	No
17-Ketogenic with bismuthate	No	Yes	Yes

Fig. 17.6 Relation of side-chain structure to analytical reactions for corticosteroids and their metabolites.

Importance of Blood Levels. The presence in blood of an endocrine constituent that is active upon injection is frequently taken as an "acid-test" of its actual hormonal role, showing that it is actually released from its site of origin. Undoubtedly some of the steroids found in the adrenal gland are synthetic intermediates and do not escape in significant quantities. Here again the dimension of *flux* or *turnover* must be introduced to give blood analyses full significance. Neither the blood level nor the glandular level alone of a hormone tells us reliably about the relative rates of its release by the gland. For example, a lowered glandular content could result either from an accelerated release or a slowed synthesis.

If all a hormone must pass through a given pool (for example, the amount present in the blood stream), if we know *the size of that pool* and either the *removal* or *renewal rate,* and if the pool does not change in size during the experiment, we can calculate the rate of endogenous release of the hormone.

Although the blood represents our best opportunity to sample the pool through which the steroids must pass, the pool concerned is not simply the whole blood volume; instead it includes considerable extravascular steroid, as will be shown for hydrocortisone in the following section. A similar conclusion was evident in the preceding chapter with reference to the hormonal iodine pool. Because blood chemistry is emphasized so much, we are apt to forget that there are very few instances where the pool we are sampling is limited to the circulating fluid.

Measurement of Steroids in Blood. Such methods mostly have exacting requirements and so far have not attained general clinical utility. The Silber-Porter method has been adapted to the determination of the 17-hydroxycorticosteroids in plasma. Hydrocortisone has been found to account for at least 80% of the reactive steroid extractable by chloroform. One shortcoming of blood hormone analyses is emphasized by the finding that the plasma 17-hydroxycorticosteroid level is subject to regular 100% diurnal variations (Samuels). If blood is always collected at a standard time, the range of normal values is not excessively wide. Nevertheless plasma analyses overlook possible hour-to-hour variations in hormone release, whereas the analysis in urine of a characteristic excretion product *integrates the production rate* over the urine collection period. On the other hand, changes in excretion rates may also reflect decreased renal clearance or slowed hepatic disposition of a steroid.

State in the Blood. The steroid hormones are not very soluble in water, although it is true that they are absorbed via the portal route upon feeding (as shown for testosterone and hydrocortisone), in contrast to the much more insoluble fats. Their "solubility" is greatly increased by adding protein to a saline solution and is much greater in plasma. Most of the steroid hormone present in plasma is not ultrafilterable and obviously is protein-bound.

On the other hand, steroids can usually be extracted from serum as completely by solvents that do not denature proteins as by those that do, which speaks against a tight combination with protein. No evidence has been obtained for an enzymatic step in the combination; instead this appears to occur spontaneously when steroids are added to plasma. The less polar steroids become more strongly bound than the more polar corticosteroids.

Dialysis experiments show that at physiological levels of hydrocortisone, it is bound fairly tightly by a rather unstable globulin fraction. At higher concentrations this protein becomes saturated with the steroid, and a protein with a lower affinity binds about three-quarters of additional steroid, up to at least a level 0.12 mg. per 100 ml. (Bush). The latter binding is probably by serum albumin. The protein-binding is qualitatively and quantitatively different after glucuronate conjugation of the steroid (Roberts, Szego).

The character of the protein-binding described in the preceding paragraph indicates that the effective concentration ("chemical activity," "chemical potential," or "escaping tendency") of the steroid varies in a nonlinear way with its total plasma level. At the level where the tight-binding proteins become saturated, the escaping tendency of the steroid increases disproportionately as its total level is raised.

The hormone that appears at the highest level in plasma is not necessarily the one most rapidly transferred to target cells. Information remains unsatisfactory at this writing concerning such transfer. Erythrocytes appear to fix added hydrocortisone avidly; this is true also after their lysis. At the moment we do not know whether cells have barriers to steroid penetration, whether penetration is actually necessary for hormone action, or whether instead there may be specific transport systems for facilitating steroid uptake. Labeled hydrocortisone, injected intravenously into a man preoperatively, entered the liver very extensively in 1 hr., but the amounts in muscle and adipose tissue were no larger than could be explained by their extracellular fluid contents (Migeon and Sandberg).

Rate of Removal from the Plasma; Rate of Release of Hormone. Studies of this type may be illustrated by experiments performed by Peterson and Wyngaarden. A tracer amount of 4-C^{14}-hydrocortisone was injected intravenously; at serial intervals the specific activity of the plasma hydrocortisone, separated by paper chromatography, was measured. An initial rapid decline of specific activity, lasting 20 to 30 min., was taken to represent the time required for equilibration with the vascular and extravascular portion of a miscible hydrocortisone pool. This pool turned out, upon extrapolation to zero time, to represent roughly two to five times the amount of hydrocortisone present in the blood stream. Undoubtedly it includes hormone present at an unknown level in the interstitial fluid where the extent of protein-binding is not known, and it may include hydrocortisone at other sites, perhaps in the adrenal cortex. The half-life of the hydrocortisone in this pool was found to average 83 min., corresponding to an endogenous rate of removal of hydrocortisone from the pool of 17 to 29 mg. per day (nine normal persons). Administration of ACTH may elevate this output to as much as 250 mg. per day (Cope).

Other techniques have been applied to this problem. Perhaps one of the most valuable is the measurement of the arterio-venous differences of the hormone, across the gland where it is elaborated (Farrell). Interesting results may also be anticipated by measuring A-V differences at sites of action and sites of metabolism of hormones. These two sites may, of course, be the same if the steroid is modified structurally in exerting its action. Table 17.1 illustrates the tentative conclusions reached as to plasma levels and endogenous production rates in man for a number of steroids. Species differences are striking in glucocorticosteroid secretion: Whereas man is mainly a hydrocortisone secretor, the rat secretes largely corticosterone.

The acceleration of aldosterone release during sodium restriction is much more clearly shown by the values for the *production rate* (last column), which in this case are based on urinalysis, than by the plasma levels.

The rapid inactivation of these hormones is evident from the short turnover times shown. If a change in the rate of hormone release is to help correct a *present* disturbance and not a *past* one, the hormone must be destroyed rapidly. Accordingly these substances must be able to modify metabolism quickly before they are destroyed. The possibility has already been considered that they mod-

Table 17.1. Tentative figures for plasma turnover and production rates for some hormones. The cortical steroid levels are morning levels. (From summaries by Pearlman and by Ayres, et al., in "Hormones in Blood," Ciba Foundation Colloquium 11, 1957.)

STEROID	CONDITION OF SUBJECT	PLASMA LEVEL, $\mu g/100$ ml.	PLASMA TURNOVER TIME	PRODUCTION RATE, mg./day
Progesterone	Late pregnancy	2–15	3.3 min.	250
Estriol	Late pregnancy	4	ca. 6 min.	85
Estradiol	Late pregnancy	0.2		5
Estrone	Late pregnancy	0.2		20
Hydrocortisone	Normal	Avg. ca. 10	26–47 min.	17–29
Aldosterone	Normal	Mean 0.08	. . .	0.18
Aldosterone	Sodium restriction	Mean 0.14	. . .	0.78

ify metabolism by being converted to something else. Hepatic conjugation with glucuronate appears to be one of the faster modes of disposition; the resulting inactive glucuronide may be present in the circulation as an analytical complication.

In summary, plasma analyses emphasize the dynamic state of plasma steroids and should provide a description of the steroidal environment of the cell, but for the time being, urinary estimates of the rate of hormone production may be more valuable diagnostically.

LIBRARY PROBLEMS

1. Are the direct metabolic effects of hormones completely restricted to the target organ? Discuss the proposition that *target reactions* rather than *target tissues* will eventually be found. Consider how a hormonal effect on a widely distributed metabolic reaction could have a more critical influence in one tissue than on another.
2. Characteristically our knowledge of the actions of a hormone passes through a stage where a confusing variety of diverse and apparently unrelated effects are attributed to the hormone. Illustrate this situation for a specific hormone. In the long run we expect that at the actual secretory levels, only one or possibly two direct chemical actions of a hormone will be found. Discuss why the confusing intermediate stage is almost inevitable.

3. Experiments by Daughaday, using paper electrophoresis under some conditions, gave equivocal results as to which plasma protein binds hydrocortisone. Why may electrophoresis be a rather unreliable way for demonstrating loose protein-binding of a substance?
4. Examine case 43051, *New England J. Med.*, **256,** 223 (1957). Discuss how each of the following were ruled out: primary aldosteronism; excessive serotonin production; pheochromocytoma. How might a determination of the urinary 17-ketosteroid or 17-hydroxycorticosteroid excretion have assisted the diagnosis? Why were pituitary gland changes expected and sought?
5. Examine the report of case 43481, *New England J. Med.*, **257,** 1086 (1957). Comment on the response of the 17-ketosteroid and 17-hydroxycorticosteroid excretion rates to the administration of ACTH; to the administration of a fluorine-bearing hydrocortisone analog.
6. Are the three methods for adrenocorticalsteroids *specific* to these substances, or are there other compounds that will give closely related color reactions? To what extent does their specificity depend upon the color reaction per se, and to what extent to preliminary separation procedures for the steroids? Why is hydrolysis by β-glucuronidase preferred?
7. Norymberski describes both a *direct* and an *indirect* method for determining 17-ketogenic steroids. Show how these differ.
8. What effects can be anticipated on the plasma and urine corticoids from a drug that specifically inhibits 11-β-hydroxylation of steroids? Is such an agent available?

18

HEME PRECURSORS AND PRODUCTS

The iron content of a normal adult man has been estimated at 4 to 5 g., about three-quarters of this in heme form, mainly hemoglobin and myoglobin but also including the heme enzymes. Storage iron is estimated at nearly one-quarter of the total and circulating nonhemoglobin iron at 0.1%.

An adult man ordinarily needs to absorb only about a milligram of iron per day because the organism so efficiently re-uses the iron released by heme breakdown. Every day somewhat less than 1% of the red blood cells break down and their hemoglobin is destroyed, the average life of the red cell being about 125 days, but only a tiny fraction of the roughly 25 mg. of iron released is lost. A woman needs to absorb more iron because of losses by menstruation, pregnancy, and lactation. The fetus acquires 200–400 mg. of iron from the mother during intra-uterine life. During the first three or four months of post-natal life, the infant absorbs little iron, relying upon iron received transplacentally earlier (Smith, et al.).

The iron economy of the body is unusual in being controlled by its *absorption* rate. The organism is believed to be protected normally from the hazard of excessive absorption of food iron by an arrangement known as the *mucosal block*. The *mucosal block* is believed to reside in the mucosal content of apoferritin, an iron-binding protein, and its relative saturation with iron to form ferritin (Granick). In general, when the demand for iron is increased (for example, in hypochromic anemia or pregnancy), this mucosal mechanism is considered to permit the rate of iron absorption to increase.

The so-called mucosal block may fail in various situations. A maize diet, or a diet low in phosphate, especially if it is high in iron, can permit excessive iron absorption. The mechanism also may fail in pyridoxine deficiency. Pancreatic damage may also cause excessive iron absorption. The accumulation of excess body iron is shown by the presence of unusual quantities of *hemosiderin* granules, i.e., of iron-staining granules. Secondary factors govern the extent of cell damage that may accompany hemosiderin accumulation.

Iron Transport. The iron of the mucosal ferritin is transferred to the metal-binding globulin, *siderophilin,* in the plasma. This protein can bind two iron atoms per molecule of 90,000 molecular weight. The iron in the plasma is in the ferric form, and normally the iron-binding capacity (about 300 to 350 μg. per 100 ml. of plasma) provided by this protein is only about one-third saturated; i.e., the plasma iron is normally about 106–143 μg. per 100 ml. (males) or 94–122 μg. per 100 ml. (females). The degree of saturation of the iron-binding protein is an important guide to the iron economy of the body, being low in iron deficiency and high in hemosiderosis and in hemolytic, pernicious, and hypoplastic anemias. An exception to this rule appears to occur in the anemia of infection, where administered radioactive iron is diverted to "storage," whereas the serum iron remains low (Greenberg).

Research into iron metabolism in disease illustrates the tendency to substitute *flux* measurements for *concentration* measurements. For example, the rate of iron removal from the plasma may be measured after injecting radioactive iron previously complexed to the metal-binding globulin. This rate is increased, for example, in iron deficiency and hemolytic anemias but slowed when erythropoiesis is depressed (Huff). Radioactive iron accumulation in marrow aspirates are also measured, as well as accumulations in the spleen by counting from the body surface over this organ. At the same time the average red blood cell life may be determined by labeling with Cr^{51} as chromate.

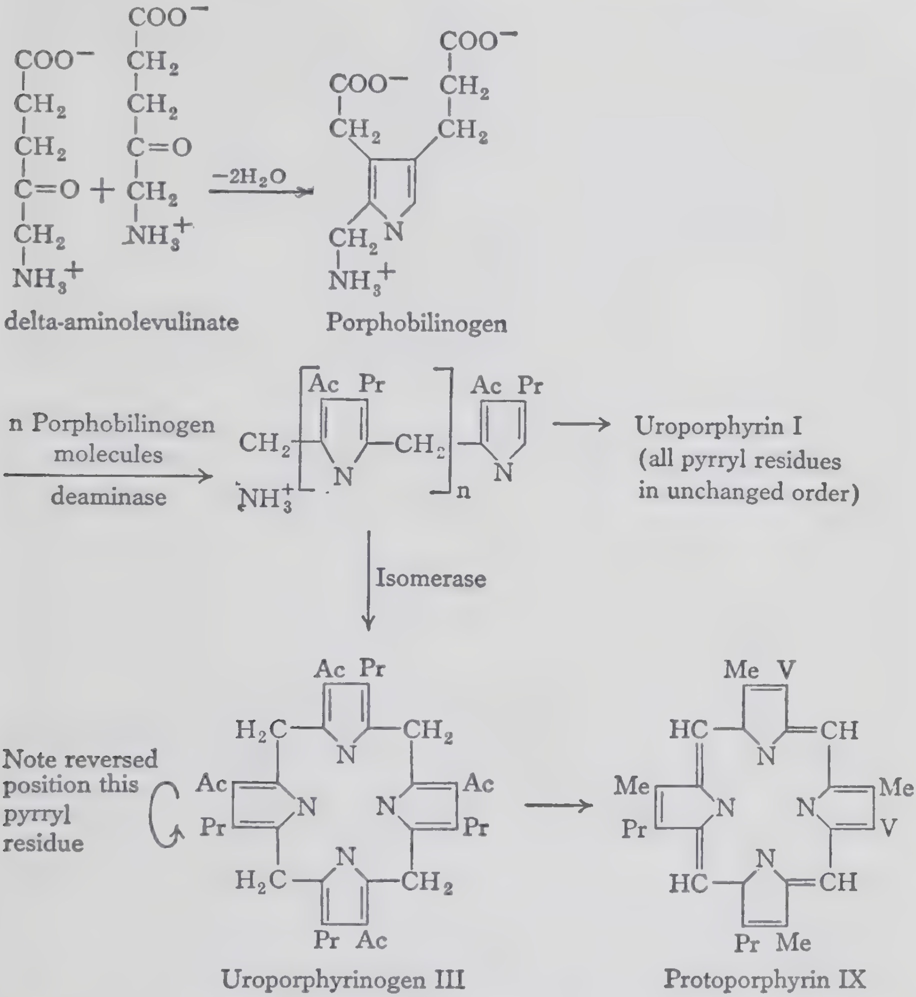
Storage of Iron. Iron is "stored" as ferritin and hemosiderin. Pure ferritin, as seen microscopically, is a beautiful orange crystalline protein. The colorless iron-free protein, apoferritin, binds the Fe as micelles of basic ferric phosphate to yield ferritin. The micelles contribute the astonishing total of 23% of iron to ferritin, higher in hemosiderin. The protein is located in the parenchymal cells of liver and spleen, in the intestinal mucosa, and in the bone marrow and in reticuloendothelial cells in general. The presence of iron stabilizes the apoferritin, which otherwise tends to break down in its absence; hence this is an *adaptive* protein, the level of which depends upon the iron supply. The release of the iron from ferritin probably occurs by its reduction to the ferrous form, in analogy to the release of Cu from ceruloplasmin. When liver cells disintegrate in hepatocellular disease, ferritin appears in the circulating blood (Reismann and Dietrich).

Hemosiderin is also a normal storage form, although ferritin ordinarily predominates. This substance may simply be ferritin that has been overloaded with iron; however, it appears to contain other proteins besides apoferritin (MacKay). Hemosiderin formation has been suggested to result when iron enters a tissue more rapidly than ferritin can be synthesized (Granick). The most recently deposited iron appears to be in a more labile pool than iron received earlier (Greenberg and Wintrobe).

Organic Precursors of Heme. Porphyrin synthesis, as far as we know, is limited to the cells in which the heme spends its life. Perhaps this fact categorizes the subject as cellular biochemistry rather than distributional; we need, however, to consider this pathway to understand the origin of porphobilinogen, the distribution of which does have present diagnostic importance.

Under the catalytic action of a pyridoxal phosphate enzyme system, glycine combines with "active succinate," probably succinyl coenzyme A, to yield an intermediate that decarboxylates to delta-aminolevulinic acid. Two molecules of δ-aminolevulinic acid then combine enzymatically to form porphobilinogen (Shemin). A deaminase is believed to catalyze the polymerization of several porphobilinogen units into polypyrrylmethanes in which the acetate and propionate side chains alternate. If their sequence is not modified, the final product is the apparently useless uroporphyrin I. Normally an isomerase manages to get one out of four of the pyrryl units inverted, so that its propionate and acetate residues are in reversed order. Only if this happens are the correct uroporphyrin III and protoporphyrin IX structures produced, the methyl and vinyl groups of the latter arising from acetate and propionate side chains, respectively. A small amount of type I porphyrin is also produced.

In *congenital porphyria* a backing-up of the pools of δ-aminolevulinic acid and porphobilinogen occur. The latter often appears in the urine, along with a greatly increased amount of type I porphyrin. This porphyrin is not degraded to bile pigments; its excessive formation also leads to a troublesome porphyrin deposition in the body. Deposits in the skin lead to the photosensitivity



−2H₂O
delta-aminolevulinate
Porphobilinogen
n Porphobilinogen molecules
deaminase
Ac
Pr
n
Uroporphyrin I (all pyrryl residues in unchanged order)
Isomerase
Note reversed position this pyrryl residue
Me
V
Uroporphyrinogen III
Protoporphyrin IX

Fig. 18.1 Suggested pathways in porphyrin synthesis. Adapted from Granick and Mauzerall, *J. Biol. Chem. 232,* 1138 (1958), omitting some intermediates.

of congenital porphyria. The occurrence of hemolytic episodes may be a consequence of the presence of type I porphyrin in the red cells. This hemolytic activity in turn accelerates heme synthesis, leading to still more type I porphyrin.

Labeling experiments with N^{15} glycine show that in normal persons, most of the label that enters porphyrins does not appear in the urine for over 100 days, indicating that it has passed through the red cell hemoglobin stage. In congenital porphyria a much larger part of this label enters the bile pigments promptly; hence it has never entered hemoglobin. Although the disease may be described as an abnormality in the enzymatic conversion of porphobilinogen to types I and III porphyrin, the accumulation of δ-aminolevulinic acid and porphobilinogen and the direct diversion to bile pigment formation remain to be explained.

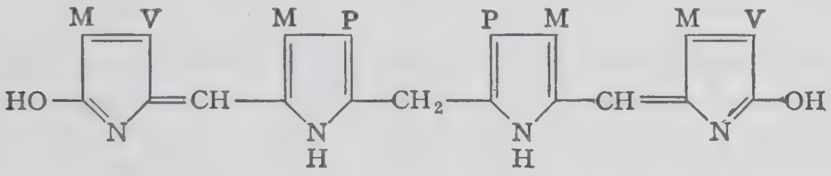
In preliminary reports Rimington notes that a porphyrin-forming enzyme preparation from erythrocytes is changed by heating at 60° so that it forms exclusively the symmetrical uroporphyrin I. Furthermore the same enzyme preparation from congenital porphyric patients is said to yield unusual proportions of uroporphyrin I without heating. Thus an enzymatic defect in the patterning of porphobilinogen into porphyrin appears about to be uncovered. Presumably the isomerase activity (Fig. 18.1) is deficient, and with the failure of repatterning, a biologically defective series of products is produced, terminating in uroporphyrin I. The accumulation of δ-aminolevulinic acid and porphyrinogen may arise from the general slowing of the chain behind the deficient enzyme.

In *acute porphyria*, in contrast, the essential abnormality is not the accumulation or urinary excretion of porphyrins, but the excretion of porphobilinogen which, however, changes spontaneously into porphyrins when the urine stands. The biochemical lesion is therefore a different one; whether porphobilinogen formation is abnormally fast, or its conversion to porphyrin partially blocked, remains to be determined.

Diagnostic analyses include the determination of porphobilinogen in the urine with Ehrlich's aldehyde reagent and the determination of the various uroporphyrins or coproporphyrins spectrophotometrically or fluorimetrically, after suitable isolation procedures.

In *toxic* porphyrias the excretion of type III porphyrin is more prominent. Still other types of porphyria have been described.

Heme Breakdown. Over-aged red cells are phagocytized by macrophages and the porphyrin skeleton of the heme opened at one of the methine carbons, this carbon atom being lost apparently as carbon monoxide (Sjöstrand). Perhaps one can liken this to the opening of a necklace with loss of the clasp. This opening appears to occur before separation from the globin, yielding the intermediate choleglobin (Lemberg). The globin presumably breaks down to permit reutilization of its amino acids. The bile pigment first observed from heme breakdown is the green *biliverdin.* Within the reticuloendothelial cells the central methine group of biliverdin is reduced to a CH_2 group, forming bilirubin:



This pigment enters the plasma, where like so many other substances, it binds itself to the serum albumin. The amount of bilirubin present can be estimated crudely from the intensity of the orange-yellow color it gives to the serum (icterus index), or more specifically by the violet-colored coupled product (azobilirubin) that results when it reacts with diazotized sulfanilic acid. A portion of the bilirubin was early recognized in many cases to react slowly with this reagent; in this case the color yield could be increased by adding alcohol or acetone. The bilirubin reacting in the first minute in the aqueous system was named the *direct* bilirubin, whereas the additional amount reacting upon adding the organic solvent was called the *indirect* bilirubin. The *indirect* form was recognized as the main one accumulating in *hemolytic* jaundice, whereas the *direct* form was also increased in *obstructive* jaundice. Van den Bergh observed that the bilirubin present in the bile reacted as *direct* bilirubin.

Various highly hypothetical explanations appear in older textbooks, for example, to the effect that changes in protein binding account for the modification of one bilirubin form to the other. These explanations have persisted despite demonstrations that both forms were albumin-bound and that chemical differences remained *after* extraction from the proteins. The bilirubin giving the *indirect* reaction has been shown to be identical with *crystalline bilirubin* in solubility behavior and to give the same type of azo derivative. The *direct-reacting* pigment, however, is much more water-soluble than bilirubin and gives a separable azo derivative which has been shown to be a *diglucuronide derivative* by enzymatic and acid hydrolysis (Billing, Cole and Lathe; Schmid). The direct Van den Bergh reaction, therefore, is largely attributable to a *bilirubin diglucuronide,* its more ready reaction in water solution presumably being related to a high water-solubility. Perhaps for a related reason this is the form that appears in the urine. Minor quantities of other conjugates are not excluded from consideration.

The hyperbilirubinemia of the newborn and also congenital hyperbilirubinemia appear to arise from a defect in the conjugation of bilirubin with glucuronic acid (Lathe; Schmid). Because *free* bilirubin is neurotoxic, the symptoms of *kernicterus* are associated with its accumulation. An attempt has been reported to accelerate the conjugation reaction by feeding glucuronic acid (Danoff).

Presumably, with the new knowledge of the two forms of serum bilirubin, new methods can be devised that will distinguish between them more sharply than the direct and indirect Van den Bergh reaction.

Products of the Intestinal Modification of Bilirubin. The bilirubin excreted via the bile into the intestine is modified by microorganisms to *urobilinogen,* which is a collective term for mesobilirubinogen and stercobilinogen, colorless substances that are subsequently oxidized to *urobilin* and *stercobilin.* The latter give the feces most of its color. In the absence of hepatic or biliary disease, assay of these compounds (usually 200 to 300 mg. are excreted per day) provides a measure of the rate of heme breakdown. A portion of the urobilinogen is reabsorbed from the intestine and again excreted, mainly into the intestine. This represents the so-called enterohepatic circulation of bile pigment.

Total biliary obstruction prevents the entry of bilirubin into the intestine and hence stops all urobilinogen formation. The absence of urobilinogen in the urine therefore is taken to reflect complete obstruction. On the other hand, liver disease with only partial or intermittent obstruction can cause an unusual proportion of the reabsorbed urobilinogen to be re-excreted in the urine. In this case the urobilinogen serves as a test substance for which the hepatic and renal clearance mechanisms compete.

Bilirubin occasionally is administered exogenously to measure the rate of its hepatic clearance. The synthetic indicator *bromsulfalein* serves more conveniently for estimating the excretory function of the kidney. About 10% to 15% of an injected dose of bromsulfalein is removed per minute. The usual procedure is to draw blood samples at selected intervals and to estimate photocolorimetrically the concentration of the remaining dye. A dose of 2 mg. per kilogram is essentially all eliminated in 30 min.; only about 6% of a 5 mg. per kilogram dose remains at 45 min. Because the rate of clearance is dependent upon the hepatic blood flow, a decreased flow as well as impaired hepatocellular function may produce abnormal retention. The results of the bromsulfalein test, in contrast to the Van den Bergh test, do not reveal whether retarded clearance is caused by poor removal from the plasma or by biliary regurgitation.

LIBRARY PROBLEMS

1. Can you detect by reading how the breakthrough was made on the question of the difference between the two types of bilirubin between 1952 and 1956? Billing and Lathe have published a review on bilirubin and jaundice. Considering the relative difficulty in isolating and determining the structure of, say, insulin or penicillin, comment on the lateness of the determination of the structure of the direct-reacting serum bilirubin.
2. Investigate in detail the history, since Van den Bergh developed his test in 1913, of the various theories to explain the difference between the direct- and indirect-reacting bilirubin. Note that analysis for as many as four categories of bilirubin has sometimes been advised: immediate direct, delayed direct, and so on.
3. Consider the question: Will the careful observation of the results of perhaps three or four carefully selected tests of liver function contribute more to diagnostic skill than the more diffuse use of a "battery"

of twice or three times as many tests related to the function of this organ?

4. On the basis of reading, diagram the possible synthetic pathway by which bilirubin glucuronide is formed.
5. Examine the discussion of case 41341, *New England J. Med.*, **253,** 335 (1955). How may chronic idiopathic jaundice be differentiated by laboratory methods from obstructive jaundice? A summary of documented cases of this disease has been published.
6. Assuming that 275 mg. of bile pigment in various forms (calculated as bilirubin) are formed from red cell hemoglobin per day, calculate the average life of the red blood cell. Assume 900 g. of hemoglobin are contained in an adult man.
7. Examine case 44131, *New England J. Med.*, **258,** 652 (1958). Discuss the state of the iron in tissues, the status of the serum iron, and the origin of the excess iron. Is the fasting blood sugar level a related finding? The cerebrospinal fluid sugar level? How can the construction of a splenorenal venous shunt contribute to subsequent neurologic symptoms? Were the results of any of the other chemical analyses abnormal?
8. Find one to three important papers showing that experiments on plant porphyrins have helped in discovering the enzymatic pathway on which the defect of congenital porphyria lies.

19

GETTING USEFUL CHEMICAL LABORATORY RESULTS

Why Analyze Blood? Most biochemical events occur in the tissue cells, so that the blood is a rather unpromising place to look for changes. It is not strange that for many diseases (for example, neoplastic disease in general) no clearly diagnostic blood change has yet been discovered. We operate under a great handicap, compared to the animal experimenter, in being still largely restricted to a few body fluids as biopsy samples. When a metabolic change occurs in the cells, the event may or may not be reflected in the blood. If it is reflected there, we may or may not have a diagnostic test.

First of all the change must be *large enough*. How large is large enough? Statistically the plasma amino acid level is diminished by about 25% during pregnancy, but this fact unfortunately gives us no pregnancy test because the ranges for *normal* and *pregnancy* overlap too much. Similarly the *average* serum cholesterol is undoubtedly decreased in hyperthyroidism, but Fig. 19.1 shows that this analysis can give little assistance in diagnosing this disease because of the wide ranges of both the euthyroid and hyperthyroid levels. In Fig. 16.1 a somewhat more favorable situation was illustrated by the modest overlap between the normal range and the hyperthyroid range of the serum protein-bound iodine. The most favorable cases are those where the ranges are narrowest and the overlap minimal; for example, the normal range of serum sodium levels is so narrow that a 5% increase or decrease is likely to have a clear significance.

Secondly the change must be *specific enough*. Measurement of a concentration change that occurs as regularly as a temperature elevation would probably not be worth the effort involved. At the other extreme one of the *most* specific of serum analyses is for the acid phosphatase activity in reference to prostatic disease. Furthermore it has been possible to increase even further its specificity by use of differential inhibitors. Phosphatases maximally active at *alkaline* pH accumulate in the serum in both bone disease and liver disease (probably not the same enzyme), but a routine discrimination has not yet been achieved. Fortunately both disease possibilities are only rarely presented by the same patient. We have also seen that blood levels may be changed by totally different mechanisms (for example, slower exit, faster entrance, higher level of a binding agent) and that an analysis will not show by which pathway the level has been changed.

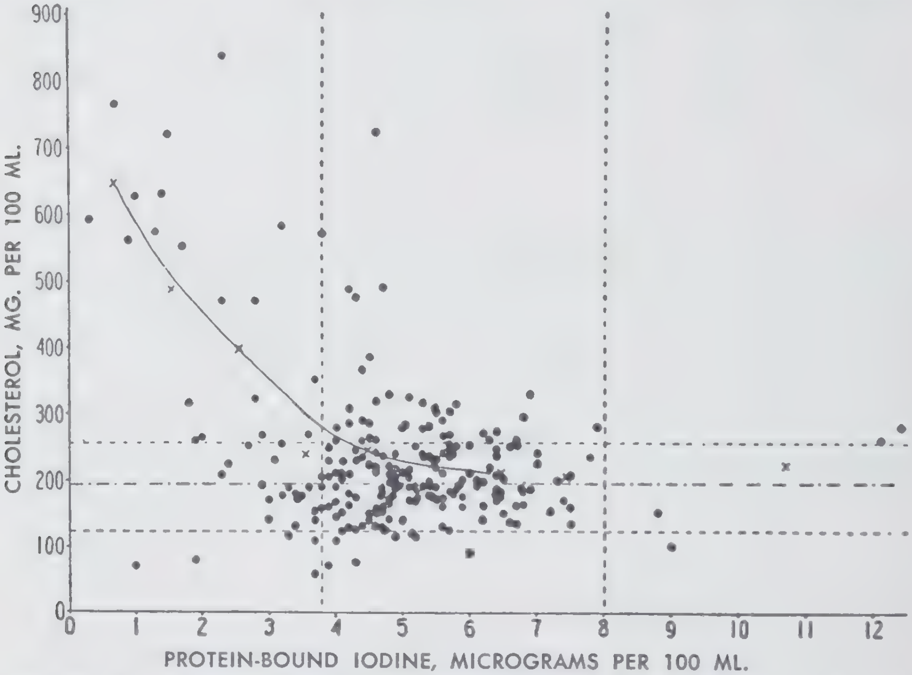


Fig. 19.1 Relationship between the serum cholesterol and protein-bound iodine levels. Each point represents a subject. The dotted lines indicate accepted normal ranges. Although there is a crude relationship between the results of the two determinations, note that most of the hypercholesteremic patients are not in the *hypothyroid* range of PBI, nor are most of the hypocholesteremic subjects in the *hyperthyroid* PBI range. (From Peters and Man, *J. Clin. Invest.*, 29, 1 [1950].)

Timing and the State of the Subject. Many blood solutes are subject to changes with meals or intensified activity. Only in the fasting or *post-absorptive* state is the level of the blood sugar, for example, fairly constant. The best time for sampling the blood, when special considerations do not apply, is early in the morning, before breakfast, with the patient in a quiet state. Furthermore the economical operation of a laboratory requires that all the samples to which a particular analysis is to be applied must be available at the same time. These two facts together mean that as many samples as possible should be secured in the early morning. Obviously analyses are required occasionally at odd hours on an emergency basis. The availability of the laboratory for such occasions usually depends upon the observation of regular schedules for the receipt of all other samples.

Choice of Sample. The plasma or serum, rather than the whole blood, is preferred for most biochemical analysis because it serves as a sample of the extracellular fluid to which our cells are exposed. An obvious exception is the hemoglobin analysis. Several other substances like urea and glucose can also be measured satisfactorily in the whole blood, thereby saving the effort needed to separate the serum. This is possible because they have the exceptional property of distributing themselves evenly in the water of the red cells and the plasma. A great deal of effort is thereby saved because these substances are so frequently determined. Cholesterol represents a situation that is only superficially similar; the red cells have about the same cholesterol content as the serum, but the cell cholesterol level does not respond as readily to metabolic changes as the serum level; therefore serum is the appropriate sample.

A number of serum or plasma analyses are made for macromolecular constituents which are present at much lower concentrations in the interstitial fluid. In these cases serum or plasma actually is selected for its own sake and not as a sample of the extracellular fluid, and special precautions must be taken to avoid modifying the serum composition during its collection. The implication is not that the plasma is a uniquely important fluid in connection with the transport of these substances, but rather that our *normal standards apply to the plasma* or *serum* collected without excessive inspissation. The level of these components in the plasma is easily increased by excessive tourniqueting. The tendency to use the tourniquet rather vigorously probably arose because the venipuncture technique was developed in connection with serology rather than quantitative chemistry. If stagnation is produced, the O_2 and CO_2 exchange will be increased, and the pH will be lowered. In addition, if the venous pressure (and therefore the mean capillary pressure) is kept high for a few minutes, unusual quantities of water and other highly diffusible components of the plasma diffuse out through the capillaries, causing macromolecular components to be concentrated by as much as 20 or 30%. Not only are the plasma protein levels increased, but also all enzyme activities and the levels of such protein-borne constituents as cholesterol, other lipids, the bile pigments, and the hormonal iodine.

The *plasma* rather than the serum must be selected for measuring prothrombin, fibrinogen, or other components that are modified by blood-clotting. Also, one micromolecular analysis, the α-amino acid concentration, is *increased* by the proteolytic reactions associated with coagulation. Unless plasma is specifically necessary, serum is preferred for chemical analyses because no foreign material needs to be added in collecting it. Plasma samples are almost always at least slightly contaminated through the use of an anticoagulant.

The circulating blood is not a homogenous fluid, and frequently the point at which it is sampled is significant. This is obvious for the blood oxygen, slightly less so for the hemoglobin level. The glucose tolerance curve also looks very different if it is determined on arterial blood (or blood flowing from a small incision in the skin which quickly approaches the arterial composition) instead of venous blood (Fig. 19.2). Similarly, if we want to know to what P_{NH_3} the central nervous system is subjected, the venous blood may not be an ideal sample.

Avoiding Artifacts. Once we have the appropriate sample collected, the level of the desired component may be disturbed in several ways. CO_2 may be lost into the atmosphere, and the pH thereby raised. Glucose may be lost by glycolysis by blood cells or by bacterial metabolism. The acid phosphatase may be lost in a few hours simply by spontaneous denaturation of the enzyme at room temperature. Potassium may enter the serum from the red cells. Hemolysis will increase both the apparent serum potassium and serum protein levels and will also discharge phosphoric esters into the serum where phosphatase action will then increase the serum phosphate.

There is no single standard way for handling blood to avoid all such changes in components. If whole blood is to be analyzed, it should be promptly mixed by a gentle swirling motion to distribute the anticoagulant (and perhaps a preservative) and then dispatched to the laboratory. It must again be mixed before each sampling to provide the correct proportion of the cells. Blood collected for serum must be taken in a dry or oiled syringe and transferred gently to a tube for centrifuging. If the CO_2 and pH are to be determined, the blood may be passed through the needle extending under mineral oil into a centrifuge tube, thereby displacing oil. In other cases the needle is first removed. Preferably after standing not more than an hour at room temperature, the blood is centrifuged and the serum separated and kept in a stoppered tube. If organic

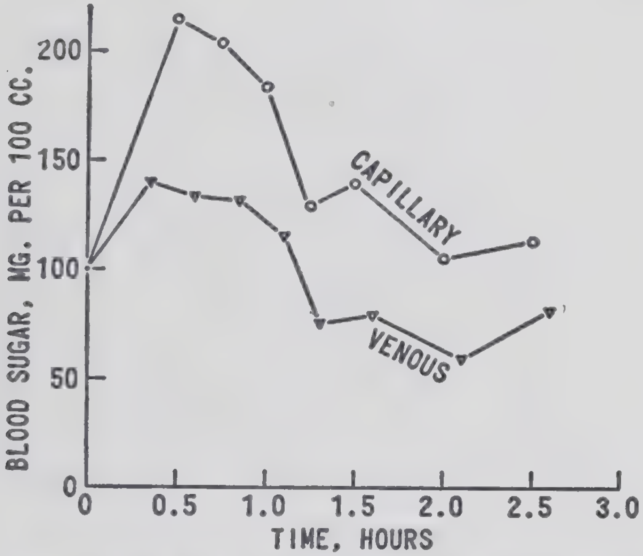


Fig. 19.2 The capillary and venous blood sugar curves of a normal young adult male after the ingestion of 100 g. of glucose. Attention is drawn to the importance of indicating whenever cutaneous blood has been used in a tolerance test. (From Foster, *J. Biol. Chem.*, **55**, 291 [1923].)

components are to be determined and delay is unavoidable, refrigeration is highly desirable.

The pH may well be determined electrometrically, using whole blood before coagulation and handling under oil. The pH obtained is, of course, the plasma pH. In any case the pH must be determined without delay. Thermostatting the electrode vessel and electrodes or the whole instrument at 37° avoids the fairly large corrections for deviation from body temperature. For this and several other determinations, blood probably should not be collected unless the laboratory analysis can proceed without delay.

Interestingly, the addition of sodium fluoride and thymol to blood can preserve the blood sugar so well that blood may be sent to a laboratory by mail in the summertime.

Deproteinization. As you look at a sample of blood, you will agree that it is a most unpromising material for microanalysis, particularly by colorimetric procedures. The serum, as well as the whole blood, tends to coagulate or become turbid upon the addition of almost any reagent. But by the deproteinization step we convert blood or serum to a clear, water-white solution, much more suitable for analysis. No single deproteinizing procedure, however, serves for all purposes.

In the Folin-Wu procedure tungstic acid is the precipitant, usually generated in the diluted blood by adding balanced quantities of sodium tungstate and sulfuric acid, in either order. The classical procedure involves combining the following:

1 vol. blood,
7 vol. water,
1 vol. 10% sodium tungstate
(5% for serum)
1 vol. $\frac{2}{3}N$ H_2SO_4 ($\frac{1}{3}N$ for serum)
—
10 vol. TOTAL

After shaking well, letting stand, and filtering, we presume that every milliliter of the filtrate contains the NPN, urea, glucose, and so on of 0.1 ml. of blood or serum.

This is the most widely used deproteinizing procedure, yielding a filtrate no more acid than pH 4 and almost free of tungstic acid (but, of course, loaded with sodium sulfate). Trichloroacetic acid, usually at a final concentration of at least 4%, yields a highly acid filtrate, providing more assurance that certain components (for example, inorganic phosphorus) remain in solution.

In the Somogyi procedure the zinc proteinates are precipitated along with excess hydrous zinc oxide. Whereas the tungstic and trichloroacetic acid filtrates contain essentially all the NPN, the Somogyi filtrate is almost free of glutathione and shows a lower although significant NPN content. It is designed for sugar analysis, glutathione and other nonsugar-reducing agents being precipitated with the proteins to permit a more specific analysis.

In the same way many other deproteinizing procedures have been designed for special purposes. For example, alcohol deproteinization is frequently preferred when serum is to be chromatographed on paper. If an enzyme is to be determined, deproteinization must, of course, wait until the enzyme has been permitted to catalyze a test reaction for a standard interval. The subsequent determination of the extent of this reaction will usually require a deproteinization, which may also serve to stop the enzyme reaction after a precise time interval.

Analysis without Deproteinization. Almost all deproteinizing agents leave a solution contaminated with added reagents; ultrafiltration is one exception. Deproteinization is omitted in several instances to avoid dangers of contamination. For example, chloride is analyzed directly by adding silver nitrate to serum and following with a boiling nitric acid digestion of organic material. The excess Ag^+ is then determined by titration. Similarly Ca is advantageously precipitated directly from serum as the oxalate. In both cases the analysis *can* be made on an appropriate filtrate, but the hazard of contamination is greater.

Analyses by flame photometry are made directly on highly diluted serum. Lipids are extracted directly from serum by suitable organic solvents. Carbon dioxide is, of course, released directly from the serum. Such colored substances as congo red, bromsulfalein, bile pigments, carotene, and ceruloplasmin may be analyzed directly in the serum with suitable control of the pH and the wave-length of observation. In addition a number of colorless substances may be determined directly by their absorption in the ultraviolet. The proteins must, of course, be determined directly in the serum or plasma unless the "deproteinization" precipitates specifically the protein to be measured; for example, fibrinogen or the globulins.

Amount of Blood. For ordinary methods one can conservatively figure that at least 2 ml. of blood should be drawn for each analysis required, or 2.5 times as much if serum is to be used. Economy in the requirement usually results if several analyses are needed. When a vein has been entered, there is usually little point in restricting the sample to less than 5 ml. even if only one analysis is needed.

The more generously equipped and staffed laboratories are able to perform analyses when necessary on considerably smaller samples, although usually at an increased expense. Single analyses can be applied in many cases to 0.1 ml. of blood or serum; still smaller volumes can be used, but the procedures become even more demanding. Such microtechniques are a valuable resource where only a small blood sample can be obtained readily, but they should usually be reserved for this situation.

Requisite Accuracy. *The Qualitative versus the Quantitative Test.* A number of useful examinations, especially of urine, are *qualitative* or more properly *semi-quantitative.* These tests are selected to have as nearly as possible the ideal degree of sensitivity so that a positive result is diagnostically significant. If the test is negative, we say, for example, "the patient excreted *no* urine protein," although this certainly is not a literally correct statement. Sometimes the methods available turn out to be *too* sensitive (for example, tests for fecal blood). Frequently as many as five grades of positivity are recorded, ±, +, ++, +++, and ++++. If these really can be recognized consistently by all observers, we are near to a quantitative method. It may be better for the observer to attach instead an equivalent numerical concentration to each analysis, thereby permitting some manipulation of the result. For example, one could then take into account how much the formation of a very concentrated urine increases the positivity of a urine sugar test. Occasionally old qualitative methods persist after new quantitative methods, which actually are easier, become available. Incidentally, if you promptly place a *quantitative* result in one of the three pigeonholes—too low, too high, or normal—you have relegated it to a *semi-quantitative* status.

This brings us to the question: *How accurate need we be* in an analysis made for diagnostic purposes? Here we encounter the human difficulty in maintaining necessary standards of accuracy and yet abhoring expensive unneeded accuracy. Shortcuts and economy of effort in analysis must be based on a thorough knowledge of the procedure and on experience as to which aspects are critical. For example, we may decide to be satisfied with an NPN value that may be 10% too high or too low. This does not mean, however, that we can "get by" with an error of 10% in measuring one of the two deproteinizing agents. Instead we may find we have made a final error of 100%! Lacking a profound acquaintance with the method and what it will bear, we had better accept each detail with respect. On the other hand the modern investigator uses many facile procedures that may seem to violate classical chemical requirements. The decision is not a rigid one but is based upon the accuracy needed in relationship to that which a given technique will certainly provide.

Inaccuracy in the clinical laboratory arises less from the selection of methods that are inherently too crude, than from failure constantly to confirm that the method is working well. Why this occurs will be considered in the following section.

Choice of Laboratory Determinations. Perhaps the quality of your training in laboratory diagnosis will be most quickly shown here. Let us propose these two valid objectives for a laboratory analysis:

1. Diagnosis and control of therapy
2. Education and research

Some questionable reasons for an analysis are these:

1. The analysis is traditional in this disease.
2. Everyone enjoys seeing a result come out as predicted.
3. Groping for a clue.
4. A superior may ask, "Was the . . . determined?"

In some teaching hospitals house-officers are encouraged to requisition numerous analyses for educational purposes, with little concern for economy of effort. This policy undoubtedly has special educational values, but it may delay the development of the selective function. It is the writer's opinion that in the soundest educational atmosphere, the requisitioning of unnecessary analyses is almost as freely challenged as the omission of a needed test. Educational purposes clearly call for the illustration of each type of diagnostic investigation for a given observer-population as opportunity offers, but they do not call for repetition of similar investigations over and over, except as required by the entrance of a new group of observers.

This position is taken with the belief that there are almost never enough laboratory services to fill all needs. If the laboratory *seems* to have enough time for its work, this may well be an illusion. First of all, most clinical chemical laboratories have not had time for many years now to perform analyses in duplicate and to include *knowns* or *controls,* so that these ideals have almost been forgotten. Secondly the physician may not understand what special "tailor-made" services an unhurried well-staffed laboratory can provide him. Special problem-solving is rarely compatible with present laboratory work loads.

Several studies have shown that the patient frequently is not receiving satisfactory laboratory services for his money. Whenever the physician feels skepticism about a laboratory analysis, he should remember that it is an *individual and collective responsibility of medicine* to protect the quality of these services. Present financial arrangements are frequently inherently incompatible with good results. Probably because clinical laboratories for many years made mostly the less demanding qualitative observations and few of the present quantitative chemical analyses, the support of both technical and supervisory personnel has always lagged far behind the salaries that industry or private laboratories find necessary to protect their decisions. The availability of dedicated technicians of high caliber, trained in the past, has often masked the deficiency, as has also the "corner-cutting" of the uncontrolled, unduplicated analysis.

A dogma among hospital administrators is that the laboratory and x-ray services may be expected to pay more than their own way. If this illusion could be abandoned as far as the laboratory is concerned, a long step would be taken in bringing biochemical observations to their full possible service to medicine.

If the analytical reports of the struggling laboratory lead to uncritical skepticism, accompanied by increased demand for more of the same, a morale problem is added to the economic one. The medical student who has himself wrestled with laboratory analyses at first may be tempted to derive satisfaction from the difficulties that others may encounter in quantitative procedures; but he should remember that he inherits the responsibility for protecting the quality of this service.

One can state in a few words, as has been tried here, some guiding principles about selecting analyses, choosing the type of sample and protecting its composition, and finally in interpreting a change of concentration. But success in these matters depends upon an understanding of the nature of the materials to be analyzed and the principles governing their metabolism and distribution.

LIBRARY PROBLEMS

1. The following are special problems that were presented to a hospital chemical laboratory for solution. Suggest in each case how the analyst might most simply have provided the answer:

    a. Fluid is issuing from the umbilicus of a patient. Will any chemical determination show whether it is serous fluid, or urine from a urachus?
    b. Fluid is issuing from the nasal passages of a child who has been struck by an automobile. Is it cerebrospinal fluid or a fluid of less dire significance?
    c. A patient received a mineral oil enema the night before his operation. Unexpectedly, at operation, a pool of free oil was found in the peritoneal cavity. Is the sample sent to the laboratory a mineral oil or an adipose fat, perhaps released by a fat necrosis?

2. The following are proposed as possible explanations for the changes in the level of blood or serum constituents in disease: (a) overproduction or underproduction; (b) overexcretion or underexcretion; (c) excessive or deficient intake; (d) excessive or deficient breakdown;

(e) abnormal distribution between cells and extracellular fluid; (f) abnormal escape into the blood stream; (g) complex or incompletely understood causation. For each of these eleven explanations select one clinical laboratory analysis that typically illustrates it. Discuss each of your selections in a sentence or two.

3. Lacking a dry syringe, an interne rinses a wet syringe with physiological saline before taking a blood sample. Which determinations will be made invalid: serum sodium, serum chloride, serum albumin? Which analysis will suffer the largest percentage error upon excessive tourniqueting, the serum albumin or the serum cholesterol? Which will be most seriously affected by partial hemolysis, the serum Na^+, the serum K^+, or the total serum protein? The whole blood NPN is slightly higher than the serum NPN. Does the inclusion of the red cells in the sample measured elevate or lower the milligrams of NPN per 100 ml. contributed by each of the following: urea? glutathione?
4. Divide a list of chemical determinations frequently made in the clinical laboratory into those which are: (a) likely to be *decreased* by chemical changes upon the standing of blood; (b) likely to be *increased* by such chemical changes; (c) not likely to be modified by such chemical events. Also mark each of the determinations that must be made specifically on (d) whole blood, (e) plasma, or (f) serum. Would plasma decalcified by a cation-exchange resin be completely uncontaminated?
5. In case 37392, *New England J. Med.*, **245,** 504 (1951), a serum calcium of 10.6 mg. per 100 ml. was interpreted to offer support to a diagnosis of hyperparathyroid disease. What factors permitted the discusser to recognize this as an elevated calcium level? What factors determine how decisively a laboratory method separates normal results from pathological results? Is *statistical significance* of a given change in disease synonymous to *diagnostic significance?* Upon what evidence was Dr. Leafs' diagnosis of tubular acidosis based? Were the tubules still able to synthesize ammonia?
6. Screen the literature superficially from 1920 to 1950 to form an idea of the clinical significance attributed to *peptidemia*. Judging from reported work, how would you rate the diagnostic *specificity* of this analysis? What is its present status?
7. Find data or make a guess as to the relative shapes of an oral amino acid tolerance curve on (a) portal, (b) peripheral venous blood. Is the hematocrit or the hemoglobin concentration constant at all points in the circulation?
8. Carbon dioxide is more soluble in mineral oil than it is in water; yet mineral oil is placed over blood to minimize CO_2 loss. Why does not the oil accelerate the loss of CO_2? What fluid is preferred for confining blood when the most precise CO_2 determinations are needed?

9. Discuss the proposition that the patient justly may be expected to pay for analyses made for teaching (or even research) purposes, if these purposes eventually contribute significant value to him through the clinical service rendered by the hospital.
10. Present a published report of a case in which you feel that substantially more chemical analyses were made than demanded by consideration of the welfare of the patient alone, or for a description of his disease.
11. A number of years ago a question worded more or less as follows appeared on an examination of the National Board of Medical Examiners: "A clinical laboratory of a hospital is faced with a shortage of personnel so that curtailment of chemical laboratory services is necessary. Name 5 chemical analyses of blood that you believe should be continued as essential. Name 5 others that are less urgent, but that should be restored as soon as possible. Explain your selections." Do you think a small hospital with inadequate support should limit the *scope* of the analyses handled in order to defend the quality of the analyses considered most necessary? How could the decision about the scope be handled? Do you think *every* hospital should limit the scope of analyses to be attempted?
12. Along the lines of the preceding question, consider what chemical laboratory procedures and equipment you would recommend for a solo practice in an area where the distance to the nearest laboratory delays any analysis by one or two days and doubles its cost. For example, do you think a filter photometer (about $200) should be obtained?
13. Find and report a study of the *accuracy* of laboratory analyses made in a group of hospitals.
14. Examine a handbook of normal values for various clinical laboratory analyses. (Various pharmaceutical laboratories provide such booklets.) See if you can find in the booklet (a) internal inconsistencies; (b) an ambiguous basis of designating a result; (c) bases of designation that seem undesirable to you; (d) normal ranges that are of little value because the method used must be known but is not indicated. Compare with the list referred to following Problem 15.
15. Explore what qualitative and semi-quantitative chemical tests may now be made at the bedside by the use of premixed powders or pretreated papers. Estimate the diagnostic place deserved by these tests. Do you think that such bedside methods will eventually replace quantitative laboratory procedures? If opportunity permits, observe the operation of an automated analytical method and discuss the implications of this development.

For the assignments 16, 17, and 18 reference should be made to a published list of the methods used and the normal ranges accepted by a leading hospital (*New England J. Med.*, **254**, 29 [1956]).

16. Why may the use of any preservative be contraindicated when urine is to be examined for nonglucose reducing substances? Do you think the value of *zero* cited in the article above as normal for such substances is literally correct? Why should iodoacetic acid be added if blood lactate or pyruvate are to be determined? What are the hazards in preserving a blood sample and a urine sample, respectively, for ammonia determinations? When total urinary N is to be measured (for example, in determining the N balance), a quantity of sulfuric acid frequently is placed in the urine bottle as a preservative. Why is this effective?

17. Which of the following substances are actually measured in serum, and which are calculated from other analytical values?

    "Indirect" bilirubin
    Globulins
    Albumin
    Cholesterol esters
    Neutral fat
    Prostatic acid phosphatase

18. Suppose a house-officer uniformly collects part of each blood sample in a clean dry tube (to permit coagulation and separation of serum) and part in an oxalate-coated flask. For how many of the analyses listed in the article cited following Prob. 15 would neither of these two samples be satisfactory?

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APPENDIX

EXAMINATION ITEMS IN DIAGNOSTIC BIOCHEMISTRY

These items are offered for study use, under the belief that some of the decisions involved in answering them will strengthen a working comprehension in this area. Answers, which may not be unequivocally or permanently correct, are offered at the end of this section. A larger, older list of similar items covering biochemistry in general is available to departments of biochemistry and medicine on request to the author.

PART I

Below are groups of lettered headings followed by several numbered items. By placing the appropriate letter in the blank, indicate which of the lettered headings is directly associated with each numbered item.

A. Thymol blue, $pK_1 = 1.5$; $pK_2 = 8.9$
B. Bromcresol green, $pK = 4.7$
C. Phenolphthalein, $pK = 9.7$
D. None of the above

( ) 1. Ideal for determination of titratable acidity of urine
( ) 2. May occasionally be suitable for the determination of the pH of urine
( ) 3. Suitable for the titration of $0.1N$ HCl in the presence of NH_4Cl
( ) 4. Usually suitable for determining colorimetrically the pH of normal gastric juice
( ) 5. Suitable for determining the strength of a KH_2PO_4 solution by titration with standard NaOH

A. An indicator with a pK of 4.7
B. An indicator with a pK of 1.5
C. An indicator with a pK of 9.7
D. An indicator with a pK of 7.0

( ) 6. Unsuitable in the titration of $0.1N$ HCl
( ) 7. Suitable in the titration of $0.1N$ HCl but not in the titration of $0.001N$ HCl
( ) 8. Useful in the determination of pH of $[HPO_4^{=}]/[H_2PO_4^{-}]$ buffers (pK_2' for H_3PO_4, 6.8)
( ) 9. Suitable in the titration of the second $H+$ of H_3PO_4 but not if NH_4+ is also present
( ) 10. Useful in the pH determination of $[CO_3^{=}]/[HCO_3^{-}]$ buffers (second pK' of $H_2CO_3 = 9.76$)

A. Concentration of ionized Ca in the circulating blood plasma
B. Concentration of un-ionized Ca in the circulating plasma
C. Concentration of $PO_4^{\equiv}$ in the circulating plasma
D. Concentration of Na+ in the circulating plasma
E. None of these

In a normal person:

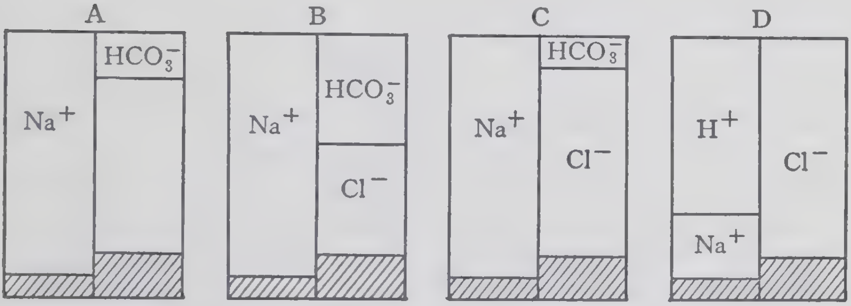
( ) 11. Tends to vary directly with the concentration of the plasma proteins
( ) 12. Important to the coagulation of blood
( ) 13. This is most sharply increased by a rise in the pH of blood
( ) 14. Amplitude of the heartbeat tends to vary with this, within a reasonable range
( ) 15. Tends to be decreased when the parathyroid hormone is injected
( ) 16. Likely to be increased if aldosterone is administered

A. Addison's disease
B. Cushing's syndrome
C. Ordinary rickets
D. Hypoparathyroid tetany
E. None of the above

( ) 17. Is accompanied by an elevated serum potassium concentration
( ) 18. Is accompanied by an excessive loss of sodium in the urine
( ) 19. Is frequently accompanied by a lowered serum inorganic phosphorus concentration
( ) 20. Is often accompanied by an alkalosis
( ) 21. Is generally accompanied by an elevated serum calcium concentration
( ) 22. Is generally accompanied by a seriously decreased extracellular fluid volume

A. 2.0
B. 4.0
C. 8.0
D. 16.0
E. None of the above

( ) 23. The serum calcium concentration, in milliequivalents per liter, for a serum sample containing 8.0 mg. per cent Ca (atomic weight Ca = 40)
( ) 24. Within the normal limits for the concentration of serum potassium, in milliequivalents per liter
( ) 25. The Hb concentration, in millimoles per liter, of a blood sample with an oxygen capacity of 17.9 vol. per cent
( ) 26. The inorganic P in millimoles per liter of a serum sample containing 3.1 mg. per cent P (atomic weight P = 31)
( ) 27. Number of isomers possible having the structure, $CH_3 \cdot CHOH \cdot CHNH_3{+}COO{-}$
( ) 28. The charge on an alpha particle



E. None of these

( ) 29. Approximates normal serum electrolyte relationships
( ) 30. Serum electrolyte relationships suggesting severe metabolic acidosis
( ) 31. Electrolyte composition most nearly representing that of pancreatic juice
( ) 32. Serum electrolytes suggesting metabolic alkalosis
( ) 33. Electrolyte composition most nearly representing that of the gastric juice
( ) 34. If vomiting of acid gastric juice deranges the electrolyte relationships of serum, this situation is the most likely

	$[HCO_3^-]$ mE./l.	$[H_2CO_3]$ mM./l.	pH
A.	40	1.3	7.3
B.	26	1.3	7.4
C.	32	2.0	7.3
D.	20	1.2	7.3
E. None of the above			

( ) 35. Internally consistent data for serum corresponding to a metaboli acidosis

( ) 36. Internally consistent data for serum corresponding to a respirator acidosis

( ) 37. Internally consistent data corresponding to a normal situation

( ) 38. Not internally consistent for serum

( ) 39. Internally consistent data corresponding to a compensated alkalosi

A. An unusually high urinary bicarbonate is indicated
B. An unusually high urinary P probably will be found
C. There must be an analytical error!
D. A high NH_4+ is likely
E. If any excess H+ is being excreted, it must be in the form of NH_4+
F. None of the above statements applies

A 24-hr. urine from a human subject shows:

( ) 40. A pH of 6.2 and a titratable acidity of 50 milliequivalents per day

( ) 41. A pH of 4.8 and a titratable acidity of 26 milliequivalents per day

( ) 42. A pH of 7.4 and a titratable acidity of 2.6 milliequivalents per day

( ) 43. A pH of 6.2 and a titratable acidity of 20 milliequivalents per day

( ) 44. A pH of 7.4

( ) 45. A titratable acidity of minus 40 milliequivalents per day

A. Less than 1
B. 27
C. 32
D. 37
E. 69
F. More than 70

A 24-hr. urine sample (vol., 850 ml.) has a pH of 4.8. The titratable acidity is 32 milliequivalents; the whole sample contains 37 milliequivalents of ammonium ion, 34 millimoles of inorganic phosphorus, and 105 milliequivalents of chloride. pK_2 for phosphoric acid = 6.8; log 2 = 0.3; log 3 = 0.48; log 4 = 0.6. In the foregoing example, nearest approximation to:

( ) 46. Number of milliequivalents of $H+$ excreted through the ability of the kidney to acidify the urine
( ) 47. Total number of milliequivalents of $H+$ excreted through various special renal activities
( ) 48. Milliequivalents of *free* $H+$ excreted in the urine
( ) 49. Milliequivalents of bicarbonate excreted in the urine
( ) 50. Number of milliequivalents of titratable acidity accounted for by the inorganic phosphate present

The blood urea N will

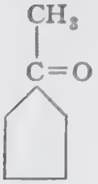
A. Steadily increase as time goes on
B. Steadily decrease as time goes on
C. Increase to a significantly higher value
D. Decrease to a significantly lower value
E. Remain substantially constant

( ) 51. If the urea clearance is reduced to one-third of normal
( ) 52. If a well-nourished adult doubles his protein intake
( ) 53. As an individual becomes progressively dehydrated
( ) 54. If an individual fasts without becoming dehydrated

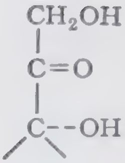
A. Pregnanediol glucuronide
B. 17-ketosteroids
C. Glucuronides
D. None of the above

( ) 55. Amount excreted in urine can be used to evaluate androgen production
( ) 56. Amount excreted in urine useful to indicate normal progress of pregnancy
( ) 57. Amount excreted in urine may be useful in estimating adrenal steroid production
( ) 58. Urinary content likely to be increased whenever the excretion of almost any steroid is increased, or when certain drugs are given
( ) 59. Amount excreted in urine measures stilbestrol secretion

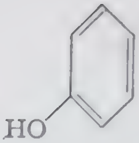
A.



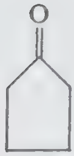
B.



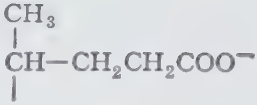
C.



D.

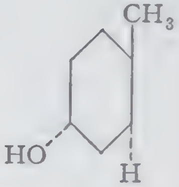


E.



$\overset{CH_3}{\overset{|}{\underset{|}{CH}}}-CH_2CH_2COO^-$

F.



G. None of the above

The numbered items refer to the steroids, fragments of whose structures are shown in (A), (B), (C), (D), (E), and (F).

( ) 60. Will reduce Benedict's solution and give a color reaction with phenylhydrazine

( ) 61. A feature producing a useful color reaction, the Zimmerman reaction, with *m*-dinitrobenzene

( ) 62. Important to fatty acid absorption

( ) 63. An 18-carbon steroid

( ) 64. Functions to maintain quiescence of the uterine musculature in pregnancy

( ) 65. Stimulates the development and vascularization of the uterine endometrium

( ) 66. May be either an androgen or estrogen

( ) 67. May well have an oxygen at C_{11}

( ) 68. Not part of any naturally occurring steroid hormone

A. Thyroglobulin

B. Free dialyzable thyroxine and triiodothyronine

C. Thyroxine and triiodothyronine bound to proteins by peptide linkages

D. Thyroxine and triiodothyronine, in loose association with a serum protein

E. Iodide

( ) 69. Form in which iodine first enters the thyroid gland

( ) 70. Main form of iodine in the "colloid" of the thyroid

( ) 71. What we measure in serum when we determine its "protein-bound iodine"

( ) 72. Form in which the thyroid hormone is transported by the circulatory system

A. Excessive use of tourniquet in collecting blood
B. Loss of a constituent from blood due to improper preservation
C. Hemolysis
D. Inadequate deproteinization
E. Use of potassium oxalate as anti-coagulant
F. There is no reason for suspecting that this result may be unreliable

Most plausible conclusion in each of the following situations:

( ) 73. The serum K+ was reported to be 10 milliequivalents per liter, although the patient had a normal electrocardiogram
( ) 74. The blood sugar was reported to be 28 mg. per cent, although the patient was in no distress
( ) 75. A blood sample taken from a normal person showed a pH of 8.05
( ) 76. The serum NPN was 80 mg. per cent; the urea N was 10 mg. per cent; the uric acid and creatinine levels were normal
( ) 77. A plasma sample showed no measurable calcium content
( ) 78. The laboratory found no acid phosphatase in the serum. Subsequently at operation a carcinoma of the prostate gland, with metastases to the pelvis, was found

A. Inadequately cleaned apparatus
B. Incorrect balance between two reagents used
C. Inadequate mixing
D. Failure to wash one of reagents used from his product
E. Not an abnormal result for the method used

The most probable reason that the following difficulties were encountered in biochemical analysis is

( ) 79. A tungstic acid filtrate of blood has a brown turbid appearance
( ) 80. In a urea determination using urease, no ammonia is released
( ) 81. Determining serum albumin by the Howe method (Na_2SO_4) 20% more albumin is found than is really present
( ) 82. In a determination of the urinary ammonia N, using the prescribed amount of Nessler's solution, a turbid solution is obtained

A. Electrometer
B. Geiger-Müller counter
C. Scintillation counter
D. Mass spectrometer
E. Cyclotron
F. None of the above

( ) 83. Useful in measuring stable isotopes
( ) 84. Useful in producing radioactive isotopes
( ) 85. Useful in separating stable from radioactive isotopes
( ) 86. Measures the energy dissipated by radiation
( ) 87. Most practical for measuring C^{14}
( ) 88. Most useful for measuring gamma radiation
( ) 89. Light flashes actually are counted by it

PART II

Below are two lettered headings and the words "both" and "neither," followed by several numbered items. If only one of the two lettered headings pertains closely to a numbered item, insert in the blank the appropriate letter, A *or* B. *If both lettered headings pertain, insert the letter* C. *If neither pertains, insert the letter* D.

A. Phenolphthalein, $pK = 9.7$
B. Bromcresol green, $pK = 4.7$
C. Both
D. Neither

Useful indicator if:

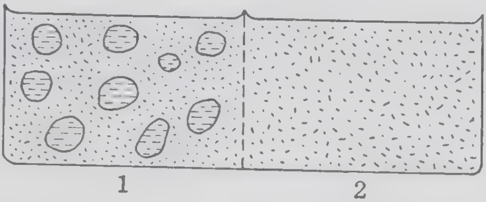
( ) 90. One wishes to titrate HCl without interference by NH_4Cl, which is also present
( ) 91. One wishes to determine colorimetrically the pH of gastric juice (pH usually between 1 and 3.5)
( ) 92. One wishes to determine the concentration of H_3PO_4 by titration (pK's 2.0, 6.7, 12.7)
( ) 93. One wishes to titrate pure glycine in solution to determine its concentration
( ) 94. One wishes to determine the concentration of lactic acid ($pK = 3.9$) by titration
( ) 95. One wishes to determine the pH of urine samples in the range 5.5 to 7.5

A. Optical density
B. Transmittance
C. Both
D. Neither

( ) 96. Directly proportional to the concentration of the color-yielding substance, according to Beer's law
( ) 97. Directly proportional to wave length of light used
( ) 98. A zero value is assigned to the reference blank
( ) 99. Directly proportional to the amount of radiant energy passing through the solution
( ) 100. For a blue solution, will be higher if a blue filter is used than if a red filter is taken

The diagram illustrates two compartments separated by a cellophane membrane. A 0.1N NaCl solution is placed in both compartments. Then solid sodium ribonucleate is added to compartment 1 and dissolved. The big polyvalent ribonucleate anions cannot pass through the membrane, but Na^+, Cl^-, and water can pass through.

A. Sodium ion
B. Chloride ion
C. Both
D. Neither



( ) 101. Net movement will be from compartment 1 to compartment 2
( ) 102. Net movement will be from compartment 2 to compartment 1
( ) 103. At equilibrium its concentration will be higher in compartment 1 than in compartment 2
( ) 104. At equilibrium its concentration will be higher in compartment 2 than in compartment 1
( ) 105. Net movement will be in same direction as net water movement

A. Extracellular fluid
B. Cellular fluid
C. Both
D. Neither

( ) 106. Organic anions are the most abundant type of anion
( ) 107. At least 40% of total osmotic pressure contributed by anions
( ) 108. Magnesium over 5 milliequivalents per kilogram
( ) 109. Potassium is permanently sealed into this compartment, except in disease
( ) 110. Electrolyte content significantly changed in dehydration
( ) 111. A net loss of both nitrogen and phosphorus specifically suggests depletion of this
( ) 112. Loss of this can be measured accurately by the net loss of either sodium or potassium

A. Serum calcium concentration
B. Serum inorganic phosphate concentration
C. Both
D. Neither

( ) 113. Increase ordinarily promotes calcification of bone
( ) 114. Only part of this is in a form directly involved in calcification
( ) 115. The amount of this that is in a form directly involved in calcification will depend on the pH
( ) 116. Decreased by parathyroid hormone administration
( ) 117. Tends to be decreased in late or terminal nephritis

A. Absorption of calcium from the intestine
B. Demineralization of bone
C. Both
D. Neither

( ) 118. Promoted by vitamin D
( ) 119. Promoted by an acid environment
( ) 120. Promoted by a lowered phosphate level in the environment
( ) 121. Promoted by parathyroid hormone
( ) 122. Inhibited by steatorrhea

A. Serum potassium level
B. Serum calcium level
C. Both
D. Neither

( ) 123. Increased by administering parathyroid hormone
( ) 124. Decreased by administering aldosterone
( ) 125. Troublesome increases possible without preceding increase in intake
( ) 126. Troublesome decreases possible without preceding change in intake or excretion
( ) 127. Usually decreased by elevating the serum inorganic phosphate
( ) 128. Tends to be increased when there are abnormal elevations of the serum protein concentration
( ) 129. In a deficiency of this element the extracellular fluid tends to be more alkaline than normally

A. Movement of potassium into cells
B. Movement of potassium out of cells
C. Both
D. Neither

( ) 130. Totally prevented by the nature of cell membrane
( ) 131. At least a portion of this requires the expenditure of energy
( ) 132. In normal functioning cells (for example, erythrocytes) this proceeds considerably the faster of the two
( ) 133. Tends to be stimulated by dehydration
( ) 134. Tends to be stimulated by glycogen synthesis and increased glucose metabolism
( ) 135. May participate in the development of symptoms of potassium deficiency

A. Chondroitin sulfate
B. Collagen
C. Both
D. Neither

( ) 136. Important constituent of cartilage and bone
( ) 137. Orientation of crystals of bone salt are determined by this
( ) 138. Important constituent of connective tissue
( ) 139. Macromolecular
( ) 140. Fibrous protein

A. Na+
B. K+
C. Both
D. Neither

( ) 141. Negative balances during the development of dehydration
( ) 142. Renal tubular reabsorption promoted by aldosterone
( ) 143. Tends to enter cells in dehydration or fasting
( ) 144. Tends to enter cells during treatment of diabetic coma with insulin and glucose
( ) 145. Serum level tends to be elevated in Addison's disease
( ) 146. Deficiency tends to cause extracellular alkalosis
( ) 147. Total amount in body considerably increased in ascites or edema
( ) 148. Body content of this ion can be determined by measuring its concentration in serum
( ) 149. Promotes uptake of CO_2 by blood

A. Total blood oxygen content
B. Total blood carbon dioxide content
C. Both
D. Neither

( ) 150. Most of it is bound to hemoglobin
( ) 151. A major part of it is present as a diffusible ion
( ) 152. Concentration is normally 30 to 50% greater in the blood in the aorta than in the blood in the vena cava
( ) 153. Concentration differs by only roughly 10% between the above two vessels
( ) 154. Will be greatly increased by faster and deeper breathing
( ) 155. Decrease establishes that the subject is threatened with acidosis
( ) 156. Would be decreased by letting blood stand in the open air in a beaker
( ) 157. If drawn from a vein at the level of the elbow, would be increased by a period of tourniqueting at a point nearer the shoulder
( ) 158. The level reached, for a sample of blood under a pressure of 50 mm. Hg of the gas concerned, will be higher if the pH is 7.5 than if it is 7.3

A. Lower chloride concentration
B. Lower bicarbonate concentration
C. Both
D. Neither

Compared with the normal blood plasma:

( ) 159. Gastric juice
( ) 160. Interstitial fluid
( ) 161. Pancreatic juice
( ) 162. Saliva, pH = 6.5
( ) 163. The glomerular filtrate, an ultrafiltrate of plasma

A. Decreased plasma pH will result
B. Decreased plasma CO_2 content will result
C. Both
D. Neither

( ) 164. Subject places bag over face so that he rebreathes the same air for several minutes
( ) 165. Subject voluntarily breathes very rapidly and deeply
( ) 166. A tourniquet encircling the arm tightly is left on for several minutes. Blood is drawn from a vein below the tourniquet
( ) 167. A blood sample is left exposed in a beaker 30 min.
( ) 168. 20 g. of $NaHCO_3$ are eaten
( ) 169. 10 g. of NH_4Cl are eaten

A. Pulmonary correction of metabolic acidosis
B. Renal correction of metabolic acidosis
C. Both
D. Neither

( ) 170. Produces an ultimate excretion of excess H+
( ) 171. While this is occurring, gaseous CO_2 is being excreted more rapidly than CO_2 is being formed by metabolism
( ) 172. While this is occurring, any previously high rate of bicarbonate excretion is decreased
( ) 173. Usually involves a change in the form of excretion of inorganic phosphate
( ) 174. Usually involves a change in the form of excretion of N derived from catabolism
( ) 175. Ordinarily does not return the blood pH to 7.4
( ) 176. By definition, cannot assist during the development of a respiratory acidosis
( ) 177. Tends to lower the serum total CO_2
( ) 178. Markedly handicapped in dehydration
( ) 179. Ability to produce this is directly related to the "alkali reserve"
( ) 180. Tends to increase the "alkali reserve"
( ) 181. Illustrated by the alleviation of diabetic acidosis through catabolism of the accumulated acetoacetate resulting from insulin administration
( ) 182. Precisely measured by the urine titratable acidity

A. Titratable acidity of urine
B. NH_4^+ excretion
C. Both
D. Neither

( ) 183. Important part of the excretion of excess H^+ from the body
( ) 184. Likely to be high when the urine pH is high
( ) 185. Will be necessarily increased if a patient doubles his rate of inorganic phosphate excretion, the urine staying all the while at pH 6.0
( ) 186. Likely to be increased after the ingestion of 10 g. of NH_4Cl
( ) 187. Likely to be low if the urine HCO_3^- content is high

A. Acidosis
B. Alkalosis
C. Both
D. Neither

( ) 188. A high total CO_2 content of blood excludes this condition
( ) 189. Can be produced by excessive breathing
( ) 190. Uniformly the alveolar CO_2 pressure will be decreased from normal
( ) 191. Blood pH usually normal in this condition
( ) 192. Ordinarily, a decreased amount of secondary phosphate ion will be found in the urine in this condition

For a 24-hr. urine:

A. High titratable acidity, high ammonium content, low pH
B. High titratable acidity, low ammonium content, low pH
C. Both
D. Neither

( ) 193. Probably a response to an acidotic trend of the organism
( ) 194. Result may suggest loss of a renal activity
( ) 195. Probably means that a metabolic alkalosis is threatening
( ) 196. Typical response of an animal that has first been made potassium-deficient and then given a dose of KCl

A. Blood NPN
B. Blood urea N
C. Both
D. Neither

( ) 197. A level above 45 mg. per cent in a patient means kidney disease
( ) 198. Can be measured by the amount of ammonia it yields under specific conditions
( ) 199. All that filters through the glomerulus is unabsorbed and enters the urine
( ) 200. A Kjeldahl-type digestion with hot sulfuric acid and a catalyst is involved in its determination
( ) 201. Concentration in serum is lower than concentration in red cells

A. The maximum urea clearance, C_{max}
B. The standard urea clearance, C_s
C. Both
D. Neither

( ) 202. (Concentration of urea in urine/concentration of urea in blood) × milliliters of urine secreted per minute, provided the urine output is in a suitable range
( ) 203. It varies directly with the rate of urine secretion
( ) 204. Average normal value is 75 ml. of blood per min.
( ) 205. If 90 ml. of urine are excreted during 1 hr., this is the clearance to be calculated

A. Albumins
B. Globulins
C. Both
D. Neither

( ) 206. Their solubility is first increased and then decreased by increasing the salt content of the solution
( ) 207. Can be caused to precipitate from solution by dialysis against pure water
( ) 208. Negatively charged in normal blood plasma
( ) 209. Antibodies are included in this group in plasma
( ) 210. Largest single source of colloidal osmotic pressure of plasma
( ) 211. Includes principal lipoproteins of plasma

A. Abnormal plasma proteins in multiple myeloma
B. Abnormal urinary proteins in multiple myeloma
C. Both
D. Neither

( ) 212. Of the two, this class is excluded as precursor of the other
( ) 213. Apparently readily lost through the nephron
( ) 214. Precipitated by warming the fluid containing it to 40° to 50°C., but redissolves as the temperature approaches 100°
( ) 215. Remarkable diversity of structure demonstrated

A. Ferritin
B. Ceruloplasmin
C. Both
D. Neither

( ) 216. Normal circulating metalloprotein
( ) 217. Metallic ions present must be reduced before any can be released
( ) 218. Enzymatic activity demonstrated
( ) 219. Contains porphyrin structure

A. Glycogen with abnormally short outer chains
B. Glycogen with abnormally long outer chains
C. Both
D. Neither

( ) 220. Characteristic of Von Giercke's disease
( ) 221. Characteristically present when there is a deficiency of the debranching enzyme
( ) 222. Characteristically present when there is a deficiency of the branching enzyme
( ) 223. Structure will have an unusual proportion of one-to-six glucosidic bonds

A. Somogyi-Nelson blood sugar method
B. Fiske-SubbaRow phosphorus method
C. Both
D. Neither

( ) 224. Depends upon reduction of complexes of molybdic acid with phosphoric or arsenic acid to colored products
( ) 225. These complexes are *reduced directly* by the substance being determined
( ) 226. The factor limiting color formation is the amount of the acid that complexes with molybdic acid
( ) 227. The factor limiting color formation is the amount of the reducing agent present

A. Unesterified fatty acids in plasma
B. Unesterified cholesterol in plasma
C. Both
D. Neither

( ) 228. Characteristic rise in liver disease
( ) 229. Associated with certain globulins
( ) 230. Bound to albumin
( ) 231. A product of action of lipoprotein lipase or clearing factor

A. Epinephrine
B. Vasopressin
C. Both
D. Neither

( ) 232. A peptide hormone
( ) 233. Administration tends to increase the blood pressure
( ) 234. Administration tends to raise the blood sugar level
( ) 235. Acts to stimulate contraction of the uterus
( ) 236. Certain amino acids are biosynthetic precursors

A. Thyroxine output
B. Thyrotrophin output
C. Both
D. Neither

( ) 237. Directly stimulated by elevation of the output of the other hormone mentioned
( ) 238. Directly inhibited by elevation of the circulating level of the other hormone mentioned
( ) 239. Uninfluenced by the output of the other hormone mentioned

A. Epinephrine
B. Thyroxine
C. Both
D. Neither

( ) 240. Derived metabolically from tyrosine
( ) 241. Increases directly the active phosphorylase, for example in liver
( ) 242. Injection will promptly increase the blood sugar level
( ) 243. Exists in the plasma in a form bound by peptide linkage to protein
( ) 244. Output is increased by a specific hormone of the anterior pituitary

A. Bile pigments
B. Bile salts
C. Both
D. Neither

( ) 245. Merely excretory substances in the bile
( ) 246. Derived from cholesterol
( ) 247. A porphyrin ring contained in the structure
( ) 248. Important to fat absorption

A. Radioactive isotope
B. Stable isotope
C. Both
D. Neither

( ) 249. Can be used as tracer
( ) 250. Occur naturally
( ) 251. Concentration can be measured by Geiger counter
( ) 252. Concentration can be measured by mass spectrometer
( ) 253. N^{15}
( ) 254. Usefulness as tracer depends upon ability of the cell to differentiate from the naturally abundant form of the element
( ) 255. Atomic number differs from that of the naturally occurring form of the same element

A. Direct-reacting bilirubin in the Van den Bergh
B. Indirect-reacting bilirubin in the same test
C. Both
D. Neither

( ) 256. Reaction retarded because of stable protein-binding
( ) 257. Reaction accelerated by conjugation to a more soluble substance
( ) 258. Level rises characteristically in hemolytic jaundice
( ) 259. More characteristically excreted into the urine

A. Result reported will be too high
B. Result reported will be too low
C. Result reported will be affected in an unpredictable way
D. Result reported will not be significantly disturbed

A student makes the following "mistakes" in carrying out analyses; indicate the effect upon his results:

( ) 260. In determining the concentration of a NaOH solution of unknown concentration by titrating a standard HCl solution with it, he transfers part of the NaOH into a wet flask before pouring it into his buret
( ) 261. In the procedure of item 24, he pipets 20.00 ml. of the standard HCl into a wet flask preparatory to titrating it with NaOH
( ) 262. He supplies a wet flask to receive an acid solution, the concentration of which is to be determined
( ) 263. He supplies a wet test tube to receive a buffer solution whose pH is to be determined
( ) 264. In a photometric analysis he reads the standard 5 min. after mixing the reactants, but for the unknown the reading is delayed another half-hour

A. Unesterified cholesterol of plasma
B. Unesterified fatty acids of plasma
C. Both
D. Neither

( ) 265. Present mainly in unbound form in the plasma
( ) 266. Mainly bound to plasma albumins
( ) 267. Increased in fasting subject by growth-hormone administration
( ) 268. A product of pancreatic lipase action
( ) 269. Normally about 26 to 30% of the total serum cholesterol

A. Acetyl coenzyme A → progesterone
B. Progesterone → glucocorticoids
C. Both
D. Neither

( ) 270. Reactions occurring in the adrenal cortex
( ) 271. ACTH stimulates in the indicated sequence of reactions
( ) 272. Metabolic defects in congenital adrenal hyperplasia are believed to lie in this sequence
( ) 273. The formation of dehydroisoandrosterone in the adrenal cortex may be placed in this sequence of reactions

A. Congenital porphyria
B. Acute porphyria
C. Both
D. Neither

( ) 274. Excess porphobilinogen appears in the urine
( ) 275. Excessive uroporphyrin I formed in the body
( ) 276. Whereas the urine contains increased quantities of porphyrin, this is mainly formed spontaneously *in the urine*
( ) 277. Accompanied by jaundice

A. Serum bilirubin
B. Serum chloride
C. Both
D. Neither

( ) 278. Deproteinization by tungstic acid usually precedes the measurement
( ) 279. Amount found may be increased by excessive use of the tourniquet
( ) 280. Cells should be removed from the serum rather promptly

A. Hyperadrenocorticalism associated with hyperplasia
B. Hyperadrenocorticalism caused by the presence of an adrenal tumor
C. Both
D. Neither

( ) 281. 17-Ketosteroid excretion is usually increased
( ) 282. The 17-ketosteroid excretion is generally strongly decreased by injecting cortisone in substantial doses for several days
( ) 283. Inborn enzyme defects in steroidogenesis responsible for some types
( ) 284. Excretion of 17-ketogenic compounds uniformly decreased

PART III

In the following items two quantities are to be compared. Place in the parentheses the letter A *if the first is larger. Insert the letter* B *if the second is larger. Insert the letter* C *if the two are equal or very nearly so.*

( ) 285. Sensitivity with which the presence of a blue compound in solution can be measured photometrically
A. Using light that has first passed through a blue filter
B. Using light that has passed through a filter of the most suitable color other than blue

( ) 286. A. Intensity of blue color of bromcresol green ($pK' = 4.7$) at pH 8
B. Intensity for the same solution at pH 9

( ) 287. A. Urinary Na excretion by a normal person on a diet of restricted salt content
B. Urinary Na excretion by a person with Addison's disease on the same diet

( ) 288. A. Normal fecal excretion of calcium
B. Normal urinary excretion of calcium

( ) 289. A. Urinary phosphate concentration
B. Plasma phosphate concentration

( ) 290. A. Total P concentration of muscle
B. Total P concentration of plasma

( ) 291. In a peripheral vein
A. Oxygenated hemoglobin concentration
B. Reduced hemoglobin concentration

( ) 292. A. Concentration of physically dissolved oxygen in blood in the pulmonary artery
B. The same, in the pulmonary vein

( ) 293. A. Oxygen pressure in the interstitial fluid
B. Oxygen pressure within muscle cells

( ) 294. A. Pressure of oxygen causing 99% saturation of the oxygen capacity of blood
B. Pressure of carbon monoxide causing 99% saturation of the CO capacity of blood

( ) 295. A. The number of negative charges on the average hemoglobin molecule in blood in the aorta
B. The number of the same, in blood in a peripheral vein

( ) 296. A. Average total number of moles of O_2 that the blood of a person must transport per day
B. Average total number of moles of CO_2 that the blood must transport per day

( ) 297. A. Hydrogen ions that are taken up by hemoglobin, as a result of the pH fall occasioned by the entry of CO_2 in capillary beds
B. Hydrogen ions that are taken up by hemoglobin because of its conversion from oxygenated to reduced form in the same capillary beds

( ) 298. A. The number expressing the CO_2 content of blood in millimoles per liter
B. The number expressing the CO_2 content of blood in volumes per cent

( ) 299. A. The number of bicarbonate ions formed when a given amount of CO_2 enters whole blood
B. The number of hydrogen ions that must be disposed of under the same circumstances

( ) 300. A. Carbon dioxide transported in blood as bicarbonate ion
B. Carbon dioxide transported in blood as carbaminohemoglobin

( ) 301. For a subject with a brief acute respiratory acidosis
A. Serum CO_2 capacity
B. Serum CO_2 content

( ) 302. A. Importance of Na^+ in defending the neutrality of the body
B. Importance of HCO_3^- in defending the neutrality of the body

( ) 303. Success in eliminating H^+ from the body achieved by a person *specifically by lowering* the 24-hr. urine pH to 4.5
A. When the urine contains 40 millimoles of inorganic P and 10 millimoles of ammonia
B. When the urine contains 10 millimoles of inorganic P and 40 millimoles of ammonia

( ) 304. Success of a subject in eliminating H^+ from the body
A. By increasing the titratable acidity of the urine by 20 milliequivalents per day
B. By increasing the urinary ammonia excretion by 20 millimoles per day

( ) 305. A. The extracellular concentration of amino acids
B. The cellular concentration of amino acids

( ) 306. A. Nonprotein N in 100 ml. of normal human serum
B. Protein N in 1 ml. of normal human serum

( ) 307. A. Average normal blood urea concentration
B. Average normal urine urea concentration

( ) 308. A. Whole blood urea N concentration
B. NPN concentration for the serum from the same blood sample

( ) 309. A. Blood ammonia level found by rapid analysis
B. Blood ammonia found after blood stands 2 hr.

( ) 310. A. Probable value for normal blood urea concentration
B. Probable value for blood urea in severe liver disease

( ) 311. A. Rates of urine formation at which the standard urea clearance applies
B. Rates of urine formation at which the maximum clearance applies

( ) 312. A. Average N intake of a normal infant
B. His average total N excretion

( ) 313. A. Creatinine excretion on a given normal diet
B. Creatinine excretion on the same diet supplemented by one quart of milk per day

( ) 314. A. Fraction of the NPN represented by urea N normally
B. Fraction of the NPN represented by urea N in azotemia

( ) 315. A. Percentage increase of the serum uric acid level in gout
B. Percentage increase of the miscible pool of uric acid in gout

( ) 316. A. Rate of uric acid synthesis in a normal person
B. Rate of uric acid synthesis in gout

( ) 317. Energy obtained in animals by metabolic oxidation of
A. Nitrogen
B. Phosphorus

( ) 318. A. Contribution of proteins to total osmotic pressure of serum
B. Contribution of sodium ion to the same

( ) 319. A. Solubility of albumin in pure water
B. Solubility of globulin in pure water

( ) 320. Apparent albumin found in a given serum sample
A. After precipitating the globulins with sodium sulfate according to the Howe method
B. By electrophoretic analysis

( ) 321. A. Colloid osmotic pressure due to 1 g. of serum globulin
B. Colloid osmotic pressure due to 1 g. of serum albumin

( ) 322. A. Quantity of amino acids freed by peptic digestion of protein
B. Quantity of amino acids freed by the normal activity of the intestinal enzymes on proteins

( ) 323. A. Total N excretion of a person on a diet barely adequate in protein content, the caloric requirement being supplied largely by carbohydrates
B. Total N excretion on the same diet but with the carbohydrate content halved

( ) 324. A. Minimum volume of solution that must be injected to supply 2000 Cal. in the form of glucose
B. Minimum volume of solution that must be injected to supply 2000 Cal. in the form of a fat emulsion

( ) 325. A. Blood sugar level in peripheral venous blood
B. Blood sugar level in peripheral arterial blood

( ) 326. A. Blood sugar level in a normal individual
B. Blood sugar level after partial loss of adrenal function

( ) 327. A. Blood sugar of a normal rat
B. Blood sugar of an alloxan-treated rat

( ) 328. A. Glucose concentration in plasma water in renal artery
B. Glucose concentration of glomerular filtrate

( ) 329. A. Liver glycogen level in a normal individual
B. Liver glycogen level in severe untreated diabetes

( ) 330. A. Level of protein-bound iodine normally in plasma
B. Level of protein-bound iodine expected when thyroxine-binding protein level is unusually high

( ) 331. A. Amount of hydrocortisone in the circulating blood
B. Amount of hydrocortisone that mixes with a tracer dose of labeled hydrocortisone injected intravenously

( ) 332. Apparent blood sugar found when deproteinization is by
A. The Somogyi method (zinc sulfate + alkali)
B. The tungstic acid method

( ) 333. A. Amount of Fe absorbed from the intestine daily
B. Amount of Fe built into hemoglobin daily in the same person

( ) 334. A. Molecular weight of substance represented by "direct-reacting bilirubin"
B. Molecular weight of substance represented by "indirect-reacting bilirubin"

( ) 335. A. Serum cholesterol in a properly collected blood sample
B. Serum cholesterol in blood collected from the arm after prolonged tourniqueting

( ) 336. Inorganic phosphate found in
A. Serum from fresh, unhemolyzed blood
B. Serum from unfresh, partially hemolyzed blood

( ) 337. For a given blood sample
A. Chloride concentration found for whole blood
B. Chloride concentration found in the serum

( ) 338. A. Amount of 17-hydroxycorticosteroid found in urine by Porter-Silber method
B. Amount of hydrocortisone+corticosterone released by adrenal cortex during interval represented by the urine sample

ANSWERS

1. D
2. B
3. B
4. A
5. C
6. B
7. A
8. D
9. C
10. C
11. B
12. A
13. C
14. A
15. C
16. D
17. A
18. A
19. C
20. B
21. E
22. A
23. B
24. B
25. C
26. E
27. B
28. B
29. A
30. C
31. B
32. B
33. D
34. B
35. D
36. C
37. B
38. A
39. E
40. B
41. D
42. C
43. F
44. E
45. A
46. C
47. E
48. A
49. A
50. B
51. C
52. C
53. A
54. D
55. B
56. A
57. B
58. C
59. D
60. B
61. D
62. E
63. C
64. A
65. C
66. D
67. B
68. E
69. E
70. A
71. D
72. D
73. C
74. B
75. B
76. D
77. E
78. B
79. B
80. A
81. E
82. C
83. D
84. E
85. D
86. A
87. B
88. C
89. C
90. B
91. D
92. C
93. D
94. A
95. D
96. A
97. D
98. A
99. B
100. B
101. C
102. D
103. A
104. B
105. D
106. B
107. A
108. C
109. D
110. C
111. B
112. D
113. C
114. C
115. C
116. B
117. A
118. A
119. C
120. C
121. B
122. A
123. B
124. A
125. C
126. C
127. B
128. B
129. A
130. D
131. A
132. D
133. B
134. A
135. C
136. C
137. B
138. C
139. C
140. B
141. C
142. A
143. A
144. B
145. B
146. B
147. A
148. D
149. D
150. A
151. B
152. A
153. B
154. D
155. D
156. B
157. B
158. C
159. B
160. D
161. A
162. C
163. D
164. A
165. B
166. A
167. B
168. D
169. C
170. B
171. A
172. B
173. B
174. B
175. A
176. A
177. A
178. B
179. A
180. B
181. D
182. D
183. C
184. D
185. A
186. C
187. C
188. D
189. B
190. D
191. D
192. A
193. A
194. B
195. D
196. A
197. D
198. C
199. D
200. A
201. A
202. A
203. D
204. A
205. B
206. C
207. B
208. C
209. B
210. A
211. B
212. A
213. B
214. B
215. B
216. B
217. C
218. B
219. D
220. D
221. A
222. B
223. A
224. C
225. D
226. B
227. A
228. B
229. B
230. A
231. A
232. B
233. C
234. A
235. D
236. C
237. A
238. B
239. D
240. C
241. A
242. A
243. D
244. B
245. A
246. B
247. D
248. B
249. C
250. C
251. A
252. C

253. B
254. D
255. D
256. D
257. A
258. B
259. A
260. B
261. D
262. B
263. D
264. C
265. D
266. B
267. B
268. D
269. D
270. C
271. A
272. B
273. B
274. C
275. A
276. B
277. D
278. D
279. A
280. B
281. C
282. A
283. A
284. D
285. B
286. C
287. B
288. A
289. A
290. A
291. A
292. B
293. A
294. A
295. A
296. A
297. B
298. B
299. B
300. A
301. B
302. B
303. A
304. C
305. B
306. A
307. B
308. B
309. B
310. A
311. B
312. A
313. C
314. B
315. B
316. B
317. C
318. B
319. A
320. A
321. B
322. B
323. B
324. A
325. B
326. A
327. B
328. C
329. A
330. B
331. B
332. B
333. B
334. A
335. B
336. B
337. B
338. B

INDEX

Italicized page numbers refer to Library Problems

Abstracting journals, 12
Accuracy
  responsibility for, 242–3
  standards of, 240–42
Acetazolamide (Diamox), 114, 116
Acetoacetate, 123–5, 132
Acetoacetyl Coenzyme A, 211
Acid-base balance
  anion-cation balance and, 128–33
  disturbances, types of, 106
  metabolic disturbances of, 97–111
  metabolic reactions and, *28*, *29*
  methods of determining, 107–11
  Na and Cl analyses and, 41–2
  renal influences on, 112–19
  respiratory influences on, 97–111
Acids and bases, weak, 16–26
Acidity, free and titratable, *28*, *29*, 116, *120*
Acidosis
  compensated, 101
  hyperchloremic, 106
  in dehydration, 119, 125
  in open-chest surgery, *111*
  in renal disease, 119, 125, 132
  metabolic, 97–111
  renal compensation of, 112–19
  renal tubular, 75–6
  respiratory, 97, 104, 106, 108, *111*
Active transport
  amino acids, 138, 139–40
  hydrogen ion, 114, *120*
  hypothetic models of, 51
  iodide, 203
  potassium and sodium, 48–52
  tracer study of, 50, 83–4
Acute nutritional failure, 37
Addison's disease
  serum Na and K in, 40
  steroid excretion in, 214, 217
  sugar tolerance in, 191
Adrenal hyperplasia, congenital, 214, 216, 217
Adrenocorticotrophic hormone, 210, 215
Adrenogenital syndrome (adrenal virilism), 214, 216, 217
Agammaglobulinemia, 161
Albumin, serum
  binding of bilirubin, 230
  binding of fatty acids, 178, 179–80, 182–3
  binding of steroids, 220
  determination, 158–60
  flexibility of structure, 166
  half-life, 164, *175*
  in nephrosis, 167
  origin of low levels, 167
  route of conversion to tissue proteins, 164–6, 173
  site of breakdown, 164
Albumin-to-globulin ratio, 159, 160
Aldosterone
  action, 40
  control of secretion, 210
  pool and secretion rate, 222
  structure, 210
Aldosteronism, primary, 56
Alkali reserve, 101
Alkalosis
  compensation of
    renal, 115
    respiratory, 105
  in ligation of alimentary tract, 122
  in potassium deficiency, 58–9
  in vomiting, 122
  metabolic, 103, 105, 106, 108
  respiratory, 97, 104, 108
Alkaptanuria, 142
Amino acids
  absorption, 136–7
  anabolism and catabolism, 138–9
  capture by liver, 138–9

- Amino acids (Cont.)
  - concentrative transfer, 138, 139–40
  - hormones and distribution of, 139
  - intravenous nutrition with, *145*
  - methods for, 142
  - nonessential, *144*
  - plasma
    - flux, 136, *144*
    - form of statement, *144*
    - in pregnancy, 140, *145*
  - precursors of milk protein, 165
  - titration in urine, *30, 121*
- Aminoacidurias
  - glycinuria, *145*
  - in cystinuria, 140
  - in the Fanconi syndrome, 140
  - in Wilson's disease, 141
  - overflow and renal, 140
- α-aminoisobutyric acid, 139, 186
- δ-aminolevulinic acid, 226–8
- Ammonia, pressure of, 154
- Ammonium ion
  - acidifying effect of ingestion, 125–6
  - blood
    - arterial and venous, 153
    - in hepatic coma, 153–4
    - methods, 155
    - origin, 152–3
  - distribution across a pH gradient, 119
  - hepatic release and binding of, arginine effect, 154, 156
  - renal synthesis and secretion, 116–19
  - titration of, 22, 23
- Amylase, 170, *175*
- Amyloid, *174*
- Androgens
  - accumulation, in hydroxylation defects, 214–17
  - structure, 207–9
- Anion-cation balance, 128–34
- Anions
  - as acids, 128
  - buffer and fixed, 128–9
- Antidiuretic hormone, 39, 40
- Apoferritin, 225–6
- Arginine, and blood ammonia, 154, *156*
- Artificial kidney, 62
- Atherosclerosis
  - and lipid transport, 181–2
  - and nutrition, *184*
- Azobilirubin, 229
- Azotemia, 147
- Base
  - as a term for cation, 128, 134
  - blood buffer, 134
  - definition, 18, 23
- Benzoic acid, and neutrality, 124, 126
- Bicarbonate ion
  - as alkali reserve, 101, 104
  - buffering activity (demonstration), 99
  - concentration changes in acidosis and alkalosis, 103, 106, 129
  - in carbon dioxide transport, 85
  - in cells, 27, 47
  - in defense of neutrality, 99–106, 114–15
  - migration, in gas transport, 92–4
  - urinary, and urine pH, 114–15
- Bilirubin
  - azo derivative, 229
  - clearance, 231
  - conjugates, 230, 231
  - determination, 229–30
  - in newborn, 230
  - intestinal modification, 230–31
  - origin, 229, *232*
  - state in plasma or serum, 230, *231*
- Biliverdin, 229
- Blood analysis, *see* Tests, diagnostic
- Blood buffer base, *134*
- Blood clotting, 169–70, *173*
- Bonds, electrovalent versus covalent, 130–31
- Bone
  - acid-base balance and, 76
  - formation and dissolution, 64, 67–72, 76, *78*
  - nature, 65
  - nucleation centers, 71
- Bromsulfalein excretion test, *29*, 231
- Buffer anions, 128–9
- Buffering and gas transport, 86–8
- Buffers
  - mixing of, 26, *28*
  - calculation of pH in, 24

- Calcinosis, 75, *77*
- Calcium
  - absorption
    - steattorhea and, 74, *77*
    - vitamin D and, 72, *77*
  - acidifying effect of ingested salts, 126

Calcium (Cont.)
  distribution, 64
  functions, 64
  in blood coagulation, 64
  in parathyroid disease, 71–2, *77*, *78*, *244*
  in thyroid disease, 72
  in uremia, 76, *77*
  protein-binding in serum, 65–6
  requirement, 73–4
Calcium phosphate in bone formation, 65, 69
Carbaminohemoglobin, 85, 90
Carbon dioxide, carbonic acid
  absorption curves, 94, 108
  analysis, 107, 244
  capacity or combining-power, 110
  forms of excretion, 115
  forms of transport, 85
  isohydric transport, 88
  solubility of, 80
  statement of concentration, 8
  total, and acid-base balance, 106–11
Carbon monoxide
  from heme breakdown, 229
  reaction with hemoglobin, *95*
Carbonic anhydrase
  in carbon dioxide transport, 85
  in hydrogen-ion secretion, 114, 120
Carcinoid syndrome, *145*
Carrier transport, *see* Active transport
Catecholamines, 188, *196*
Cations
  advantage, as cell constituents, 48
  as bases, 128, 131
Cephalin flocculation test, *173*, *174*
Cerebrospinal fluid
  chloride in, *44*
  enzymes in, *174*
  proteins, and Pandy test, *173*
Ceruloplasmin, 141, *144*, 163
Chelates
  definition, 53
  in amino acid transport, 141
  in calcium metabolism, 71, *76*
  in copper elimination, 141, *145*
Chloride
  acidifying effect, 105, 130, 131
  contribution to osmolar concentration, *44*
  diagnostic analyses, 41–5, 130
  dry storage of, *44*
  in cerebrospinal fluid, *44*
Chloride (Cont.)
  in digestive fluids, 33
  in serum and extracellular fluids, 33–43
  in sweat, *45*
  shift, 94
  statement of concentration, *43*, *44*
Cholesterol
  analysis, 181, 183, 184
  exchange among tissues, 180
  in atherosclerosis, 181
  in diabetes, 183
  in lipoproteins, 177, 180–83
  in liver disease, 183
  in nephrosis, 182–3, *184*
  protein-bound iodine and, 234
  synthesis, 211
  transport, 178–83
Choline esterase, *28*, 172
Chondroitin sulfate, 64, 71, *78*
Chromatography
  of amino acids, 142
  of proteins, 161, *175*
Chylomicrons, degradation of, 179–80
Citrate and bone dissolution, 71
Clearing factor, clearing reaction, 179–80, 182
Coagulation of blood, 169–70, *173*
Coeliac disease, 143
Concentration
  cellular and extracellular, 6, 8, 9
  forms of statement, 4–8
  osmolal, 6–7
  percentile scores for, 4
  significance, 5
Congo red test, in amyloidosis, *174*
Constants
  dissociation, 19
  solubility-product, 68
Copper in Wilson's disease, 141, *144*, *145*
Corticosteroids
  analysis, 215–19
  rate of release, 220–22
Corticosterone, 210, 215, 216, 218, 221
Cortols, cortolones, 212, 217, 218
Creatine and creatinine, 135, 149, *155–6*
Creatinine clearance, 149, *156*
Cryoglobulins, *173*
Current List of Medical Literature, 12
Cushing's syndrome
  alkalosis in, 58

Cushing's syndrome (Cont.)
  potassium in, 56
  steroid synthesis and secretion in, 214, 217
Cystinosis, 141
Cystinuria, 140
Cytochromes, *95*

Dehydration
  acidotic tendency in, 119, 125
  cellular electrolytes in, 53–4
  correction of alkalosis in, 119
  definition, 37
  in diabetic acidosis, *134*
Deproteinization
  hazard of incomplete, 146
  methods, 146, 238–9
Diabetes insipidus, 40
Diabetes mellitus
  acidosis of, 123–5
  glucocorticoids and, 189
  growth hormone and, 189
  insulin and, 185–8, 191
  serum cholesterol in, 183
  sugar tolerance in, 190–91
  thyrotoxicosis and, 189
Diet and neutrality, 124, 125
Digestive juices, 36, 37, 122
Dihydrotachysterol, 73

Edema, 40, 167
Electrolyte framework, 34, *44*
Electroneutrality, law of, 32
Enzymes, serum or plasma
  assay, *28,* 168, *174, 175*
  conference on, *175*
  diagnostic use, 171–3, *174, 175*
  form of statement of level, 5
  functional, 168
  origin of changes in, 171–3, *174*
Epinephrine, 188, *196*
Errors
  controls and duplicate analysis to detect, 241, 242
  frequency of, 242, *245*
  hemolysis and, 237
  in photometry, *29*
  responsibility for, 242
Erythrocyte
  electrolytes, 46–7, 63
  life-span, 224, *232*

Erythrocyte (Cont.)
  sedimentation rate, *175*
Estrogens
  assay in urine, 213
  atherosclerosis and, 181
  structure, 207–9
  transhydrogenases and, 212–13
  turnover and production rates, 222
Ethylenediamine tetraacetic acid, 76
Exchange-diffusion, 83–4
Extracellular fluid
  dehydration and, 37–8
  electrolytes, 33–43
  Gibbs-Donnan equilibrium and, 34, 35
  measure of volume, 42–3
  proteins of, 168

Fanconi syndrome, 75, 141
Fat, intravenous use, *184*
Fatty acids, plasma unesterified, 179–80, 182
Ferritin, 224, 225, 226
Fibrocystic disease of pancreas
  gelatin digestion in, 136–7
  sweat in, *45*
Flame photometry, *14*
Flux, 10, 11, 202, 203, 222
Formol titration, *121*
Freezing point lowering, 32, *44*

Galactosemia, 172, 192
Gases
  pressure and solubility, 7–8
  transport of, 79–95
Gastric juice
  effect of loss, 37, 122
  electrolytes, 33, 36
  pH and acidity, *28, 29*
Gibbs-Donnan equilibrium, 34, 35
Globulins
  definition, 158
  electrophoresis, 160
  gamma, 161
  lipoproteins, 177–83
  metal-binding, 163
  precipitation and determination of, 158–9
  pseudo, 158
Glucagon, 189, *196*

- Glucose
  - arterial and venous, 236, 237
  - blood, significance, 185
  - entrance into cells, 186–7
  - methods for, 194, 195
  - preservation in blood samples, 236, 237
- Glucose-6-phosphatase, 194
- Glucose tolerance test
  - adrenal steroids and, 196, 197
  - in adrenocortical deficiency, 191, *196*
  - in diabetes, 190, 191
  - inverted, 190, 191
  - liver function and, 191, *197*
  - method, 189, 190
  - venous and arterial, 236, 237
- Glucuronidase, in steroid analysis, 217, *223*
- Glutamic acid, glutamate
  - precursor of ammonia, 118, 154
  - therapy of hepatic comma, *29*, *156*
- Glutaminase, and urine ammonia, 118
- Glutamine
  - bound, in coeliac disease, 143
  - in ammonia metabolism, 117–18
  - precursor of purines, 150
- Glycine
  - plasma, during gelatin digestion, 136–7
  - titration of, 23–4
- Glycinuria, *145*
- Glycogen
  - restoration, and potassium transfer, 57
  - structure, and storage diseases, 192–4
- Glycogenolysis, 189, 193–4
- Glycoproteins, 161–2
- Glycosuria, 192, 195
- Goitrous cretinism, 199
- Growth-hormone
  - diabetogenic action, 189
  - unesterified fatty acids and, 180

- Heme
  - breakdown of, 229–30
  - co-ordination of iron and oxygen, 89
  - synthesis, 226–8
- Hemochromotosis, 163, *232*
- Hemoglobin
  - buffering, 87
  - carbamino, 85, 90
  - co-ordination positions of iron in, 89
- Hemoglobin (Cont.)
  - cyanide and, *96*
  - in carbon dioxide transport, 87–93
  - in plasma, 93
  - mean corpuscular, 81
  - reaction with oxygen, 80–83
  - relationship to K+, *95*
  - sickle-cell, 84
  - state of charge, 87, 88
  - statement of concentration, 81
- Hemolysis, as a source of error, 237, *244*
- Hemosiderin, 226
- Henderson-Hasselbalch equation, 19, 108–9
- Hepatic coma, 153–5, *156*
- Hexokinase, insulin and, 186, 187
- Hydrocortisone
  - effect on amino acid distribution, 139
  - fixation by cells and tissues, 220
  - in blood, 219, 220, 221
  - pool-size and turnover, 222
  - protein-binding, 220
  - recovery after injection, 217
  - route of absorption, 219
- Hydrogen ion
  - balance, *133–4*
  - central role, 130, 133
  - distribution among acceptors, 26
  - distribution among body compartments, 27, 58–9
  - endogenous production and consumption, 122–6
  - formation, in urea synthesis, 126
  - from oxidation of neutral sulfur, 125
  - removal, in ammonium synthesis, 116–19, 126
  - secretion of, 118–19
- Hydroxyapatite, 65
- β-hydroxybutyric acid, *126*
- 17-hydroxycorticosteroid analysis, 215–19, *223*
- Hydroxylation of steroids, and defects in, 212–13, 214–15, 216
- Hyperkalemia, 61
- Hypernatremia, 43
- Hyperparathyroidism, 72, *77*, *78*
- Hyperthyroidism, 72, 186, 201, 202, 204
- Hypoglycemia, 192
- Hyponatremia, 40, 43
- Hypokalemia, 57–9

Imidazole group, in hemoglobin, 87, 89
Indicators, acid-base, 27
Insulin
  action, 185–7, 190–91
  binding, 187
  deficiency, 123
  entry into cells, 187
  inactivation, 188
  in glucose tolerance test, 190–91
  resistance, 187, 189
  sugar transfer, 186
  zinc and, 187
Insulin-tolerance test, 191
Interstitial fluid, 36
Intestinal obstruction or bleeding, azotemia in, 147, *155*
Intravenous nutrition, *144*, *145*
Iodine
  butanol-extractable, *206*
  in contrast media, 203, 205
  method, 202–3
  organic, degradation rate, 202
  protein-bound, 199–201
  radioactive, 204, 205
  uptake by thyroid, 203–5
   thiocyanate and, 203
   thyrotrophic hormone and, 204
Iron
  absorption, 224–5
  conservation and distribution, 224
  flux methods, 225
  storage, 225–6
  transport, 225
Isohydric transport of CO_2, 88

17-Ketogenic steroids, 217, *223*
Ketone anions, ketone bodies, 124, *134*
17-ketosteroid analysis in urine
  applications, 214, 223
  in 17-ketogenic method, 217, *223*

Lanosterol, norlanosterol, 211
Library, use of, 11–13
Lipids
  absorption. 176
  transport, 176–83
Lipoproteins
  amino acid residues in, 180
  classification, 177–8
Lipoproteins (Cont.)
  clearing-reaction and, 179–80
  electrophoresis, 178
  flotation, 177, 178
  insulin-binding, 187
  interconversion, 179–83
Lipoprotein lipase, 179, 182
Liver disease
  serum alkaline phosphatase in, 172
  serum lipoproteins and cholesterol, 183–4

Macroglobulins, *173*
Macromolecular chemistry, 157
Magnesium
  deficiency, 61
  in cells, 48
  in extracellular fluid, 33
Malabsorption, 74, 143, 145, *196*
Meat-intoxication, 153
Metasteroids, 181, 214, *223*
Methemoglobin, 81
Mevalonic, mevaldic acids, 211
Mucoproteins, 161–3
Mucosal block, 141, 224
Multiple myeloma, 167–8, *173*, *174*
Myoglobin, oxygen absorption curve, 82

Neonatal tetany, 74
Nephrotic syndrome
  albumin in, 167, 182–3
  cholesterolemia, 167, 182, *184*
  iron-binding protein in, 167
  thyroxine-binding protein in, 167
Ninhydrin, analytical use, 142, *144*
Nitrogen balance, 54, *145*
Non-protein nitrogen
  form of statement and standards, 146, *155*
  in dehydration, 147, *155*
  method, 146
  protein breakdown and, 147, *155*
  significance, 135, 146–7
Norepinephrine, 188, *196*
Normal values
  caution in use of, 3, 4, 233, *245*
  list of, reference, 13
  manner of use, 3–4
Nucleic acids
  and uric acid, 150–52
  as cell anions, 47

Ochronosis, 143
Organic acids, organic anions, 30, 124
Osmolal concentration, 6, *44*
Osmotic pressure
  cellular and extracellular, 48
  colloidal (oncotic pressure), 7, 167, *173*
  total, 6–7
Osteoblasts, osteoclasts, 64
Oxygen
  absorption curves for, 82
  active transport, 83–4
  gradients of pressure, 79
  hemoglobin, reaction with, 80–83
  solubility, 7, 80
  transport in blood, 79–93

Pancreatic juice, 33
Pancreatitis, *78*, 171
Paradoxical aciduria, 119
Parathyroid hormone, 71–2
Partition, 8–10
Peptides
  in plasma and tissues, 143, 157, 166, *244*
  in urine, 140, 143
pH
  avoiding change in samples, 236, 244
  buffering of, 17–26
  calculation of, 24
  change, during gas transport, 86–94
  change, upon cleavage of substances, *28*, *29*
  change with acid and base concentrations, 25
  definition, 16
  diagnostic determination of, 109–11, 238
  gastric juice, *28*, *29*, *30*
  gradient, in bone metabolism, 70
  measure of, 27
  urine, *28*, *29*
Phenolsulfonphthalein test, *14*
Phenylalanine and phenylketonuria, 140, *144*
Pheochromocytoma, 188–9, *196*
Phosphatases, 70, 171–2, 174, *175*, 236
Phosphate
  absorption in neonatal tetany, 74
  calcium salts, 65, 67–70, 126
  clearance, in parathyroid disease, 72
  gradient, in bone formation, 70
Phosphate (Cont.)
  retention in uremia, 76, 133
  serum, and glucose metabolism, 61
  serum, state in, 67
  vitamin D and, 72
  in neutrality control, 113, *120*, 125, 132
Phospholipids, 176, 177, 180
Phosphorus
  balance, 54
  functions of, 64, 113
Phosphorylase, 70, 188, 189, 193
Photometry, errors in, *29*
pK
  change with salt concentration, 24, *120*
  definition, 19
Plasma
  definition, 6
  volume, and interstitial fluid, 36
Plasmapheresis, 165
Pool size
  concept, 5
  hydrocortisone, 221, 222
  plasma proteins, 165, 175
  steroids, 222
  uric acid, 151
Porphobilinogen, 226, 227, 228
Porphyrias, 227–9, *232*
Porphyrin synthesis, 226–9
Potassium
  active transport of, 48–52
  balance, 53
  binding, in mitochondria, 53, *63*
  cellular functions, 52
  cellular loss, in dehydration, 53–4, 56
  deficiency
    detection, 60
    origin, 55–8
    relationship to alkalosis, 58–9, 133
    renal and cardiac lesions in, 59
  exchangeability, 62
  hyperkalemia, 61–2
  in Addison's disease, 40
  in Cushing's syndrome, 56
  in diabetes, 57–8
  in diarrhea, 55
  in extracellular fluids, 52
  in familial periodic paralysis, 59
  in muscle, 47
  in primary aldosteronism, 56
  in red blood cells, 46, 62, *63*

Potassium (Cont.)
  renal excretion, 39, 40, 56, 114
Pregnanediol, 213
Pregnanetriol, 216
Protein-bound iodine
  and serum cholesterol, 234
  in thyroid disease, 201–2
  method, 202–3
  nature, 199
Proteins
  as anions in cells, 47
  "base-binding," *63*
  Bence-Jones, 166, *174*
  calcium-binding, 65–6
  cerebrospinal fluid, *173*, *174*
  chromatography, 160, *175*
  determination, 157–60
  dissociation, *29*
  electrophoresis, 160–61
  flux, 136, 164, *175*
  food, acidifying effect of, 125
  in synovial fluid, 168
  lipoproteins, 177–83
  metal-binding, 163
  route of interconversion, 164–7
  significance of total serum concentration, 158
  solubility, and salt concentration, 158–9
  steroid-binding, 219–20
  structure, 157, 166
  thyroxine-binding, 199–200
  transfer among cells, 164–7
Prothrombin, 168–70, *173*, *184*

Rates of processes, 5, 136
Reducing substances, non-sugar, 194, 195, *246*
Renal rickets, 75, 76
Renal tubular deficiencies, 75, 119
Replacement solutions, 60, 61, *63*
Resin therapy, 40–41, 62
Respiratory quotient, 92

Saliva, 37, 203, 205
Seromucoids, 161–3
Serum
  definition, 6
  true and separated serum, 94, 110
Sialic acid, in glycoproteins, 162
Sickle-cell disease, 84
Siderophilin, 163, 225
Significant decimal places, 6
Sodium
  diagnostic analyses, 41
  inertness, 131
  in serum and extracellular fluids, 34–43
  in sweat in fibrocystic disease, *45*
  renal resorption of, 39, 40, 114
  replacement by lithium, *63*
Solubility-product principle, 68
Squalene, 211
Steatorrhea, 74, 176
Stercobilin, stercobilinogen, 330
Steroids
  analysis in blood, 218–19
  analysis in urine, 215, 217, 218
  biosynthesis, 211, 216
  hydroxylation, 212–13, 214–16
  metabolic modification, 210, 212–13, 216
  production and turnover rates, 220–22
  structure and isomerism, 207–10
Steroid suppression tests, 214, 215, *223*
Storage
  as a concept, 44
  of iron, 225–6
Strontium, and fall-out, *77*
Sugar
  identification in urine, 195
  preservation, 236–7
  tolerance tests, 189–91
  transport, 186
Sulfate, production and retention, 125, 133
Sulfur, in food proteins, 125
Sulfonylureas, 188, *196*

Tests, diagnostic
  accuracy, 240–41
  automated, *245*
  avoiding chemical changes, 236–8, 244
  bedside, *245*
  choice of sample, 6, 235–6
  criteria for choice, 241–3
  curtailment, 242, *245*
  deproteinization, 238–9
  economics, 242–3, *245*
  objectives, 241–3
  size of sample, 240

Tests, diagnostic (Cont.)
  special *ad hoc*, 242, *243*
  standards for, (reference) 13, *245*
  timing, 235
Testosterone
  absorption route, 219
  and K+ distribution, 58
Tetany, 74, *77*, 108
Thiouracil, 204
Thymol-turbidity test, *173*
Thyroglobulin, *205*
Thyrotrophic hormone, 204, *205*
Thyroxine
  flux, 200–202
  mode of action, 200, *206*
  protein-bound, 199–200
  removal from plasma, 202
Thyroxine-binding protein, 167, 199–200
Titratable acidity
  of gastric juice, *29*
  of urine, *28*, 116, *120*, *121*
Titration
  curves, form of, 19–20
  of acetic acid, 17–18
  of ammonium, 22–3
  of glycine, 23–4
  of oxygenated and reduced hemoglobin, 87
  of phosphoric acid, 21–2
  purpose, 17
Toxemias of pregnancy, *145*
Transaminases, 172, *174*
Transhydrogenase action of steroid dehydrogenases, 212–13
Transport, *see* Active transport
Triiodothyronine, 198, 200, 205, *206*
Tubular acidosis, 75, 76
Tyrosine
  in alkaptanuria, 142
Tyrosine (Cont.)
  mono- and di-iodo, 199

Unesterified fatty acids, *see* Fatty acids
Urea
  ammonia synthesis and, 117, 118, 126
  blood, in liver disease, *156*
  blood, significance, 147, 155
  flux, in plasma, 136
  source of N, 118
  synthesis from ingested ammonium, 126
Urea clearance, *14*, 148–9
Uric acid
  degradation, *156*
  origin, 149–50, *156*
  pool-size and distribution, 151–2
  solubility and stone-formation, *120*, 152
  synthetic rate, in gout, 151–2
Urine
  acidification, 112–16, 119, 129
  alkalinization, 115
  formation, 39
  free hydrogen ion in, 113
  titratable acidity, *28*, 116, *120*, *121*
Urobilin, urobilinogen, 230–31

Van den Bergh test, 229, *231*
Vitamin D
  intoxication, 72, *77*
  mode of action, 72
Von Giercke's disease, 194

Water partition and transport, 31, 32
Wilson's disease, 141, *144*, *145*

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